

## COMPOUNDS FOR TARGETING

5 The present invention relates to compounds for use in the treatment of cancer. In particular, the invention provides fusion proteins that include an antibody portion directed against an antigen specific to tumour neovasculature fused to interleukin-12. Preferred fusion proteins of the invention bind particularly tightly to the target antigen and are useful for treating solid tumours.

### 10 Background

Treatment of cancer with targeted fusion proteins has shown much promise, but many problems remain. For example, antibody-targeted cytokines have shown much promise in the treatment of cancer in animal models and in some human studies, but the optimal choice of antibody/antigen, cytokine, and antibody effector function remains to be determined. For example, Gillies (US 5,650,150) described the general usefulness of cytokine fusions to complete antibodies, and the specific usefulness of antibody-IL2 fusion proteins.

20 Interleukin-12 (IL-12) is a particularly attractive cytokine for targeted immune therapy, because IL-12 stimulates a Th1 immune response, which is most effective in attacking tumour cells. IL-12 is quite toxic when administered systemically, consequently it is particularly important to direct its activity to a tumour site. Gillies *et al.* (WO 99/29732) described the usefulness of fusions of IL-12 to antibodies and also described particular techniques needed to express IL-12 fusion proteins, relating to the fact that IL-12 is a two-subunit cytokine in which one of the subunits can homodimerise. Halin *et al.*, 2002, *Nature Biotechnology* **20**:264-269 described a fusion protein consisting of a single-chain IL-12 moiety fused to a single-chain Fv (sFv) with the variable domains of L19, an antibody that binds to tumour-specific neovasculature. This latter molecule lacks the Fc region of the antibody and thus lacks all effector functions.

Even when IL-12 is fused to a targeting moiety, there is a period after the fusion protein is administered when the protein drug circulates systemically. During this period and before the drug accumulates in the tumour and disappears from the rest of the system, secondary cytokines are induced and damage results.

5

Hence, there is a need for improved means of delivering IL-12 to a tumour site within a patient.

### Summary of Invention

10

A first aspect of the invention provides a compound comprising a target specific portion and an effector portion wherein the target specific portion comprises or consists of a monoclonal antibody having specificity for oncofoetal fibronectin, or a fragment or variant thereof which retains the binding specificity for oncofoetal fibronectin of the parent monoclonal antibody and the effector portion comprises or consists of interleukin-12, or a functional fragment or variant thereof.

A characterising feature of the compounds of the invention is that the monoclonal antibody having specificity for oncofoetal fibronectin binds to a region of oncofoetal fibronectin other than the extra-domain B (ED-B) region. The ED-B region of fibronectin is a domain which, by alternative splicing of the primary RNA transcript, is either present or omitted in fibronectin molecules of the extracellular matrix (see below). Thus, the monoclonal antibody having specificity for oncofoetal fibronectin does not bind to the ED-B region (ED-B domain), although it binds to a splice variant of fibronectin (termed 'oncofoetal fibronectin') which comprises such an ED-B region.

By "target specific" portion we mean the portion of the compound which comprises one or more binding sites which recognise and bind to oncofoetal fibronectin. Oncofoetal fibronectin is a protein that is expressed by tumour cells and is associated with tumour vasculature. This protein is also expressed in foetal tissue, but does not appear to be expressed at all in normal adult tissue except for

regenerating endometrium and wound healing (Carnemolla *et al.*, 1989, *J. Cell. Biol.* **108** p1139-1148).

5 Oncofoetal fibronectin is generated by alternate splicing in tumour cells, through which an additional domain, termed the ED-B domain (complete type II repeat ED-B, also known as extratype III repeat B [EIIIB]), is inserted between fibronectin repeats 7 and 8. ED-B is a highly conserved domain with one hundred percent homology in the mammals studied to date (see Carnemolla *et al.*, 1989, *supra.* and ffrench-Constant *et al.*, 1989, *J. Cell. Biol.* **109** p903-914).

10

Thus, the invention provides compounds for delivering IL12, or a functional fragment or variant thereof, to tumour cells by targeting oncofoetal fibronectin.

15 By “specificity for oncofoetal fibronectin” we mean that the target specific portion (*i.e.* the monoclonal antibody, or a fragment or variant thereof which retains the binding specificity for oncofoetal fibronectin of the parent monoclonal antibody) binds to oncofoetal fibronectin but does not bind substantially to fibronectin expressed by normal adult tissue.

20 Suitable monoclonal antibodies to target antigens (in this case, oncofoetal fibronectin) may be prepared by known techniques, for example those disclosed in *Monoclonal Antibodies: A manual of techniques*, H Zola (CRC Press, 1988) and in *Monoclonal Hybridoma Antibodies: Techniques and Applications*, J G R Hurrell (CRC Press, 1982) and *Antibody Engineering, A Practical Approach*,  
25 McCafferty, J. *et al.*, ed. (IRL Pres, 1996).

The target specific portion of the compounds of the invention are characterised by having specificity for a region of oncofoetal fibronectin other than the ED-B region. Rather, the target specific portion binds to a cryptic epitope which is  
30 exposed/accessible in oncofoetal fibronectin (which comprises the ED-B domain) but is not exposed/accessible in normal fibronectin (which lacks the ED-B domain). As a consequence, the target specific portion binds to the splice variant

of fibronectin which comprises an ED-B domain, but does not bind to the ED-B domain itself.

Thus, targeting agents comprising the L19 antibody or antigen-binding fragments thereof (for example, as described in WO 03/076469) are excluded from the scope of the present invention, since the L19 antibody binds to the ED-B domain.

Preferably, the target specific portion binds to an amino acid sequence present in fibronectin expressed in both foetal and normal adult tissue. More preferably, the target specific portion binds to a fibronectin domain flanking, *i.e.* adjacent, the ED-B domain. Most preferably, the target specific portion binds to an amino acid sequence within the repeat 7 domain of fibronectin (see Example 3 below).

It will be appreciated by persons skilled in the art that the compounds of the invention may target oncofoetal fibronectin expressed by any species. Advantageously, the compounds are targeted to oncofoetal fibronectin from the species in which the compounds are to be used therapeutically. Thus, in a preferred embodiment, the target specific portion is specific for human oncofoetal fibronectin.

In a particularly preferred embodiment of the first aspect of the invention, the target specific portion comprises or consists of a BC1 antibody, or an antibody capable of competing with the binding of a BC1 antibody to oncofoetal fibronectin or a fragment or variant thereof which retains the antigen binding specificity of the parent monoclonal antibody. Production of the BC1 antibody is described in EP 0 344 134 B, and it is obtainable from the hybridoma deposited at the European Collection of Animal Cell Cultures, Porton Down, UK (Accession No. 88042101)

The BC1 antibody binds specifically to oncofoetal fibronectin via a site on repeat 7, outside the ED-B domain, that is masked in normal fibronectin but accessible when the ED-B domain is present (Carnemolla *et al.*, 1989, *J. Cell*

*Biol.* **109**:1139-1148; Carnemolla *et al.*, 1992, *J. Biol. Chem.* **267**:24689-24692; Mariani *et al.*, 1997, *Cancer* **80**:2378-2384; see also Example 1 below).

5 Methods for determining whether a test antibody is capable of competing with the binding of a BC1 antibody to oncofoetal fibronectin are well known in the art, such as competitive ELISA.

10 In a further preferred embodiment, the BC1 antibody is a human or humanised antibody. By 'humanised monoclonal antibody' we include monoclonal antibodies having at least one chain wherein the framework regions are predominantly derived from a first, acceptor monoclonal antibody of human origin and at least one complementarity-determining region (CDR) is derived from a second, donor monoclonal antibody having specificity for oncofoetal fibronectin. The donor monoclonal antibody may be of human or non-human origin, for  
15 example it may be a murine monoclonal antibody.

Preferably, both chains of the humanised monoclonal antibody comprise CDRs grafted from a donor monoclonal antibody having specificity for oncofoetal fibronectin.

20 Advantageously, the CDR-grafted (*i.e.* humanised) chain comprises two or all three CDRs derived from a donor antibody having specificity for oncofoetal fibronectin.

25 Conveniently, the humanised monoclonal antibody comprises only human framework residues and CDRs from a donor antibody having specificity for oncofoetal fibronectin.

30 However, it will be appreciated by those skilled in the art that in order to maintain and optimise the specificity of the humanised antibody it may be necessary to alter one or more residues in the framework regions such that they correspond to equivalent residues in the donor antibody.

Preferably, the framework regions of the humanised antibody are derived from a human IgG monoclonal antibody.

- 5    Methods of making humanised monoclonal antibodies are well-known in the art, for example see Jones *et al.* (1986) *Nature* **321**:522-525, Riechmann *et al.* (1988) *Nature* **332**:323-327, Verhoeyen *et al.* (1988) *Science* **239**:1534-1536 and EP 239 400.
- 10   In a further preferred embodiment, the compound of the first aspect of the invention binds to oncofoetal fibronectin with high avidity. By "high avidity" we mean that the target specific portion recognises oncofoetal fibronectin with a binding constant of at least  $K_d = 10^{-6}$  M, preferably at least  $K_d = 10^{-7}$  M, suitably  $K_d = 10^{-8}$  M, more suitably  $K_d = 10^{-9}$  M, yet more suitably still  $K_d = 10^{-10}$  M, and
- 15   more preferably  $K_d = 10^{-11}$  M or even  $K_d = 10^{-12}$  M.

Preferably, the compound of the first aspect of the invention binds to oncofoetal fibronectin more tightly than the parent monoclonal antibody, *e.g.* BC1, used to produce the target specific portion. The tightness with which the compound and

20   parent monoclonal antibody bind to oncofoetal fibronectin may be measured by determining a dissociation constant for binding to oncofoetal fibronectin (see Examples 2 and 3).

Advantageously, the compound binds to oncofoetal fibronectin at least 2-fold

25   tighter than the parent monoclonal antibody, for example at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20-fold tighter. Conveniently, the compound binds to oncofoetal fibronectin at least 10-fold tighter than the parent monoclonal antibody binds to oncofoetal fibronectin.

30   In a preferred embodiment, the compound of the first aspect of the invention comprises a target specific portion comprising or consisting of a whole (*i.e.* intact) monoclonal antibody, preferably a BC1 antibody. Thus, the target specific portion

may comprise two immunoglobulin heavy chains and two immunoglobulin light chains, which may be linked by disulphide bonds. One or more of the component chains may be conjugated, *e.g.* fused, to the effector portion. For example, the two immunoglobulin heavy chains may each be fused to an effector portion.

5

In an alternative preferred embodiment of the compounds of the invention, the target specific portion comprises or consists of an antigen-binding fragment of a monoclonal antibody having specificity for oncofoetal fibronectin (*e.g.* BC1).

- 10 The variable heavy ( $V_H$ ) and variable light ( $V_L$ ) domains of an antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by humanisation of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of
- 15 the rodent parented antibody (Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6851-6855).

- That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial
- 20 expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**, 1038); disulphide-linked Fv molecules (Young *et al.*, 1995, *FEBS Lett.* **377**:135-139); single-chain Fv (ScFv) molecules where the  $V_H$  and  $V_L$  partner domains are linked via a flexible
- 25 oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* **349**, 293-
- 30 299.

The advantages of using antibody fragments, rather than whole antibodies, may be

several-fold. The smaller size of the fragments allows for rapid clearance, and may lead to improved tumour to non-tumour ratios. Fab, Fv, ScFv, disulphide Fv and dAb antibody fragments can all be expressed in and secreted from bacteria, such as *E. coli*, or eukaryotic expression systems such as yeast or mammalian systems, thus allowing the facile production of large amounts of the said fragments.

Preferably, the target specific portion of the compounds of the invention comprises an antigen binding fragment of the humanised antibody selected from the group consisting of Fab-like molecules, such as Fab and F(ab')<sub>2</sub>, Fv molecules, disulphide-linked Fv molecules, ScFv molecules and single domain antibodies (dAbs).

More preferably, the target specific portion comprises a Fab molecule or a F(ab')<sub>2</sub> molecule.

In a preferred embodiment, the compound of the first aspect of the invention comprises a target specific portion comprising a human BC1 heavy chain variable region of SEQ ID NO: 1.

20

EVQLVQSGADVKKPGASVKVSCKASGYTFTNYVMHWVRQAPGQGLEWL  
GYINPYNDGTQYNERFKGRVTMTGDTSTAYMELSRLTSDDTAVYYCAR  
EVYGNYYIWGNWGQGTLVSVSS

[SEQ ID NO:1]

25

Advantageously, the compound of the first aspect of the invention comprises a target specific portion comprising a human BC1 light chain variable region of SEQ ID NO: 2.

30 EIVLTQSPGTLSPGERATLSCSASSSISNYLHWYQQKPGQAPRLLI  
YRTSNLASGIPDRFSGSGSGTDFTLISRLEPEDFAVYYCQQGSSIPFT  
FGQGTEKLEIK

[SEQ ID NO:2]

Conveniently, the compound of the first aspect of the invention comprises a target specific portion comprising a human BC1 heavy chain variable region of SEQ ID NO: 1 and a human BC1 light chain variable region of SEQ ID NO: 2.

- 5 In a further preferred embodiment, the target specific portion comprises one or more antibody constant regions, such as the CH1, CH2 and CH3 immunoglobulin constant domains. The one or more constant regions may be from same or different antibody to the variable regions of the target portion. Likewise, the compound of the invention may comprise an immunoglobulin heavy chain and an  
10 immunoglobulin light chain, each of which comprises a constant region (which constant regions may be from the same or different parent antibodies).

Preferably, the one or more antibody constant regions comprises or consists of a CH1 domain.

15

In a further preferred embodiment, the compound of the invention further comprises an immunoglobulin Fc moiety. Advantageously, the Fc moiety is derived from a human IgG1 antibody.

- 20 By “Fc moiety” we mean an antibody fragment comprising the CH2 and CH3 domains of an IgG heavy chain constant region, *i.e.* structurally equivalent to the fragment producible by papain cleavage of an IgG molecule, or a polypeptide which is functionally equivalent thereto.

- 25 As detailed above, the compounds of the first aspect of the invention comprise an effector portion which comprises or consists of IL-12 or a functional fragment or variant thereof (*i.e.* an ‘IL-12 moiety’). By a “functional” fragment or variant we include the meaning of a fragment or variant capable of stimulating a Th1 immune response in a mammalian host, *i.e.* the differentiation of Th1 cells from naïve  
30 T cells.

Thus, the effector portion comprises or consists of polypeptide having IL-12

activity.

Preferably, the effector portion comprises or consists of human interleukin-12, or a functional fragment or variant thereof.

5

Conveniently, the effector portion comprises or consists of a single-chain interleukin-12, for example comprising or consisting of an IL-12p35 domain and an IL-12p40 domain. Preferably, the IL-12p35 domain is conjugated to IL-12p40 domain by a disulphide bond.

10

Preferably, the effector portion comprises an IL-12p35 domain of the following amino acid sequence:

15 NLPVATPDPGMFPC LHHSQNLLRAVSNMLQKARQTLEFY PCTSEEDHEDI  
TKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSS  
IYEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSE  
TVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNAS

[SEQ ID NO: 3]

20

Preferably, the effector portion comprises an IL-12p40 domain of the following amino acid sequence:

25 IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTL DQSSEVLGS  
GKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLLHKKEDGIWSTDILKDQK  
EPKNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGA  
ATLSAERV RGDNKEYEYSVE CQEDSACPA AEESLP I EVMVDAVHKLKYEN  
YTSSFFIRDIKPDPPKNLQLKPLKNSRQVEVSW EYPDTWSTPHSYFSLTFC  
VQVQGKSKREKKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWA  
30 SVPCS

[SEQ ID NO: 4]

35 In a particularly preferred embodiment of the first aspect of the invention, the compound is or comprises a fusion compound or fusion protein. By “fusion compound” we include a compound comprising one or more functionally distinct portions, wherein the distinct portions are contained within a single polypeptide

chain produced by recombinant DNA techniques. For example, the compound may comprise a whole antibody wherein the heavy chain is fused to a single chain IL-12. Alternatively, the compound may comprise a Fab or F(ab')<sub>2</sub> fragment of an antibody wherein the truncated heavy chain (*i.e.* the Fd chain) is fused to a single chain IL-12.

Preferably, the target specific portion and the effector portion of the fusion compound are fused. These portions may be fused directly, or via a linker sequence (for example to allow greater flexibility of the portions relative to one another).

Suitably, the linker is a mutated linker sequence comprising or consisting of the amino acid sequence ATATPGAA [SEQ ID NO. 5].

Alternatively, the target specific portion and the effector portion of the compound of the invention are separate moieties linked together by any of the conventional ways of cross-linking polypeptides, such as those generally described in O'Sullivan *et al Anal. Biochem.* (1979) **100**, 100-108. For example, the antibody portion may be enriched with thiol groups and the enzyme portion reacted with a bifunctional agent capable of reacting with those thiol groups, for example the N-hydroxysuccinimide ester of iodoacetic acid (NHIA) or N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). Amide and thioether bonds, for example achieved with m-maleimidobenzoyl-N-hydroxysuccinimide ester, are generally more stable *in vivo* than disulphide bonds.

In a preferred embodiment, the compound comprises a polypeptide of SEQ ID NO:6

*BC1 heavy chain fused to human IL-12 p35*

EVQLVQSGADVKKPGASVKVSKASGYTFTNYVMHWVRQAPGGGLEWL  
GYINPYNDGTQYNERFKGRVTMTGDTISISTAYMELSRLLTSDDTAVYYCAR  
EVYGNYYIWGNWGQGTLSVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV

KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQT  
YICNVNHHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK  
DTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN  
STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ  
5 VYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV  
LDSGDSFFLYSKLTVDKSRWQQGNVFSVCSVMHEALHNHYTQKSATATPG  
AANLPAVATPDPGMFPCPLHHSQNLLRAVSNMLQKARQTLEFYPTSEEIDH  
EDITKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALC  
LSSIEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFN  
10 SETVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNAS

[SEQ ID NO:6]

In a further preferred embodiment, the compound comprises a polypeptide of SEQ  
15 ID NO:7.

*BC1 light chain*

EIVLTQSPGTLSPGERATLSCSASSSISSNYLHWYQQKPGQAPRLLIYRTS  
20 NLASGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQGSSIPFTFGQGTKLE  
IKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQS  
GNSQESVTEQDSKSTYSLSSLTLSKADYEKHKVYACEVTHQGLSSPVT  
KSFNRGEC

[SEQ ID NO:7]

25

In a particularly preferred embodiment, the compound comprises a polypeptide of  
SEQ ID NO:6 and a polypeptide of SEQ ID NO:7.

Advantageously, the compound further comprises a polypeptide of SEQ ID 4  
30 linked by disulphide bond to the polypeptide of SEQ ID NO:6.

Thus, the invention provides a fusion protein comprising antibody V regions  
directed against oncofoetal fibronectin, an Fc moiety, and an interleukin-12  
moiety. Specifically, the invention provides an immunoglobulin (Ig) fusion  
35 protein comprising antibody V regions that bind to oncofetal fibronectin, fused to  
interleukin-12. In a preferred embodiment of this invention, the antibody V  
regions are from the BC1 antibody (Carnemolla *et al.* (1992), *J. Biol. Chem.*

267:24689-24692; Mariani *et al.* (1997), *Cancer* **80**:2378-2384). The Fc moiety is preferably derived from human IgG1.

5 In a preferred embodiment, the fusion protein comprises antibody V regions as shown in SEQ ID 6 and 7, and an interleukin-12 moiety. Preferably, IL-12 moiety is a single-chain interleukin-12.

10 An unexpected feature of this invention is that the fusion protein binds to oncofetal fibronectin much more tightly than does the corresponding BC1 antibody alone. Such tight binding is useful in treating cancer, as the tighter binding leads to better tumour targeting of IL-12 than would be expected on the basis of the affinity of the BC1 antibody for oncofetal fibronectin. Tighter binding is particularly advantageous for target antigens that do not turn over rapidly, such as components of the extracellular matrix.

15

In a preferred embodiment, antibody constant regions are also used, *e.g.* a CH1 domain. Figure 1 illustrates some of the configurations of antibody variable regions (striped ovals), constant regions (white ovals), the IL-12 p35 subunit (small rectangles), the IL-12 p40 subunit (large rectangles), antibody hinges and linkers (thick lines) and disulfide bonds (thin lines). Particular preferred  
20 embodiments include intact IgG-type antibodies with p35 fused to the C-terminus of the heavy chain and p40 attached to p35 by a disulfide bond (Figure 1A), a 'minibody' with the antibody V regions connected by a linker and attached through a hinge to a CH3 domain, and p35 fused to the C-terminus of the heavy  
25 chain and p40 attached to p35 by a disulfide bond (Figure 1B), an sFv with p35 fused to a V region and p40 attached by a disulfide bond (Figure 1C), and an Fab with p35 fused to a C region and p40 attached by a disulfide bond (Figure 1D). The IL-12 p35 subunit may also be attached to the N-terminus of a V region. The IL-12 p40 subunit may be attached to p35 through a disulfide bond or through a  
30 linker, yielding a so-called 'single-chain IL-12' moiety (scIL-12).

In a more preferred embodiment, an intact BC1 antibody with constant regions of

human IgG1 is used. A particular advantage of this molecule is that it has effector functions such as ADCC, which are lacking in minibody, Fab, and sFv fusion proteins.

- 5 A second aspect of the invention provides a nucleic acid molecule encoding a compound according to the first aspect of the invention, or a target specific portion, effector portion or one or more component polypeptides thereof (e.g. a BC1 heavy chain, a BC1 light chain, IL12 p35 and p40 subunits and/or an Fc moiety). By "nucleic acid molecule" we include DNA, cDNA and mRNA  
10 molecules.

In a preferred embodiment, the nucleic acid molecule of the invention comprises one or more of the nucleotide sequences selected from the groups consisting of SEQ ID NOS: 8, 9 and 10.

15

*HuBC1 heavy chain fused to huIL12 p35 subunit (VH is underlined; the p35 sequence is in bold; and the upper and lower cases represent the coding and non-coding sequences, respectively):*

20 ATGGAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCTGgt  
gaggagagaggggaagtgagggaggagaatggacagggagcaggagcactgaatcccattgctcattccatgta  
tctggcatgggtgagaagatgggtcttatcctccagcatggggcctctggggtgaatactgttagagggagggttc  
cagatgggaacatgtgctataatgaagattatgaaatggatgcctgggatggtctaagtaatgccttagaagtgact  
agacacttgcaattcacttttttgtaagaagagatttttaggctataaaaaaatgttatgtaaaaaataacgatcaca  
25 gttgaaataaaaaaaaaataaaggatgttcataatgtgtataactatgtatttctctcattgtttcagCTTC  
CTTAAGCGAGGTGCAGCTGGTGCAGTCTGGGGCTGACGTGAAGAAG  
CCTGGGGCCTCAGTGAAGGTCTCCTGCAAGGCTTCTGGATACACCTT  
CACCAACTACGTAATGCACTGGGTGCGACAGGCCCTGGACAAGGG  
CTTGAGTGGCTGGGATATATTAATCCTTACAATGATGGTACTCAGTA  
30 CAATGAGAGGTTCAAAGGCAGGGTCACCATGACCGGGGACACGTCC  
ATCAGTACAGCCTATATGGAGCTGAGCAGGCTGACTTCTGACGACA  
CCGCGGTGTATTACTGTGCGAGAGAGGTCTATGGTAACTACATCTG  
GGGCAACTGGGGCCAGGGAACCCTGGTCTCCGTCTCCTCAGgtaagtaag  
ctttctggggcaggccaggcctgacctggccttggggcagggaggggggctaaggtgaggcaggtggcgccag  
35 ccaggtgcacaccaatgcccatgagcccagacactggacgctgaacctcgcgacagttaagaaccaggggg  
cctctgcgccttggggccagctctgtcccacaccggtcacatggcaccacctctcttgagCCTCCACC  
AAGGGCCCATCGGTCTTCCCCCTGGCACCTCCTCCAAGAGCACCTC  
TGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCC

GAACCGGTGACGGTGTCTGTTGGAACCTCAGGCGCCCTGACCAGCGGCG  
TGCACACCTTCCCGGCTGTCTTACAGTCTCAGGACTCTACTCCCTC  
AGCAGCGTGGTGACCGTGCCTTCCAGCAGCTTGGGCACCCAGACCT  
ACATCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAA  
5 GAGAGTTGgtgagaggccagcacagggagggaggggtgtctgctggaagccaggctcagcgtctgc  
ctggagcgcacccggctatgcagtcccagtcaggggcagcaaggcaggccccgtctgcctcttccaccggagg  
cctctgccccccccactcatgctcagggagaggggtcttctggcttttccccaggctctgggcaggcacaggctag  
gtgcccctaaccagggccctgcacacaaaggggcaggtgctgggctcagacctgccaagagccatatccggga  
ggaccctgcccctgacctaaagcccaccccaaggccaaactctccactccctcagctcggacacctctctcctcc  
10 cagattccagtaactcccaattctctctctgcagAGCCCAAATCTTGTGACAAAACCTCAC  
ACATGCCACCGTGCCCAggttaagccagcccaggcctcgcctccagctcaaggcgggaca  
gggtccctagagtagcctgcacccagggacaggccccagccgggtgctgacacgtccacctccatctcttctca  
gCACCTGAACTCCTGGGGGGACCGTCAGTCTTCTCTTCCCCCCCCAAA  
ACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCAAGTTCAACT  
15 GTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACT  
GGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGC  
GGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCAC  
CGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGAAG  
GTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCA  
20 AAGCCAAAGgtgggacccgtgggggtcgagggccacatggacagaggccggctcggcccaccctct  
gccctgagagtgaccgctgtaccaacctctgtccctacagGGCAGCCCCGAGAACCACAGG  
TGTACACCCTGCCCCCATCACGGGAGGAGATGACCAAGAACCAGGT  
CAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCC  
GTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACCTACAAGACC  
25 ACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCTCTATAGCAA  
GCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCA  
TGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGA  
GCGCCACCGCGACCCCGGGCGCCGCAAACCTCCCCGTGGCCACTC  
CAGACCCAGGAATGTTCCCATGCCTTCACCACTCCCAAAACCTG  
30 CTGAGGGCCGTCAGCAACATGCTCCAGAAGGCCAGACAAACTC  
TAGAATTTTACCCTTGCACCTTCTGAAGAGATTGATCATGAAGAT  
ATCACAAAAGATAAAAACCAGCACAGTGGAGGCCTGTTTACCATT  
GGAATTAACCAAGAATGAGAGTTGCCTAAATTCCAGAGAGACCT  
CTTTCATAACTAATGGGAGTTGCCTGGCCTCCAGAAAGACCTCT  
35 TTTATGATGGCCCTGTGCCTTAGTAGTATTTATGAAGACTTGAA  
GATGTACCAGGTGGAGTTCAAGACCATGAATGCAAAGCTTCTGA  
TGGATCCTAAGAGGCAGATCTTTCTAGATCAAAACATGCTGGCA  
GTTATTGATGAGCTGATGCAGGCCCTGAATTTCAACAGTGAGAC  
TGTGCCACAAAAATCCTCCCTTGAAGAACCGGATTTTTATAAAA  
40 CTAAAATCAAGCTCTGCATACTTCTTCATGCTTTCAGAATTTCGG  
GCAGTGACTATTGACAGAGTGACGAGCTATCTGAATGCTTCCTA  
A

[SEQ ID NO:8]

*HuBC1 light chain (VL is underlined; the upper and lower cases represent the coding and non-coding sequences, respectively)*

**ATGGAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCCTG**gtgag  
 5 gagagaggggaagtgagggaggagaatggacagggagcaggagcactgaatcccattgctcattccatgtatctggc  
 atgggtgagaagatgggtcttatcctccagcatggggcctctgggggtgaatactgttagagggagggtccagatggga  
 acatgtgctataatgaagattatgaaatggatgcctgggatgggtctaagtaatgccttagaagtactagacacttgcaat  
 tcacttttttggaagaagagatttttaggctataaaaaaatgttatgaaaaataaacgatcacagttgaaataaaaaaaaa  
 aatataaggatgttcatgaattttgtgtataactatgtatttctctcattgtttcagCTTCCTTAAGCGAAAT  
 10 TGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAG  
AGCCACCCTCTCCTGCAGTGCCAGTTCAAGTATAAGTTCCAATTACTTG  
CATTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTAT  
AGGACGTCCAATCTGGCTTCTGGCATCCCAGACAGGTTCAAGTGGCAGT  
GGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTGGAGCCTGAA  
 15 GATTTTGCAGTGTATTACTGTCAGCAGGGTAGTAGTATACCATTACGT  
TTGGCCAGGGGACCAAGCTGGAGATCAAACgtaagtggatcctatcagggttttacaaga  
 gggactaaagacatgtcagctatgtgtgactaatggtaatgtcactaagctgcgcgatcccgaattctaaactctgagg  
 gggctcggatgacgtggccattctttgcctaaagcattgagttfactgcaaggtcagaaaagcatgcaaaagccctcagaa  
 tggctgcaaagagctccaacaaaacaatttagaactttattaaggaatagggggaagctaggaagaaactcaaacat  
 20 caagattttaataacgcttcttggctctccttgctataattatctgggataagcatgctgtttctgtctgtccctaacatgccct  
 gtgattatccgcaaacacacaccaagggcagaactttgttacttaaacaccatcctgtttgcttcttctcagGAA  
 CTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTT  
 GAAATCTGGAACCTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCC  
 AGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGG  
 25 TAACTCCCAGGAGAGTGTACAGAGCAGGACAGCAAGGACAGCACCT  
 ACAGCCTCAGCAGCACCTTGACGCTGAGCAAAGCAGACTACGAGAAA  
 CACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCC  
 GTCACAAAGAGCTTCAACAGGGGAGAGTGTTAG

[SEQ ID NO:9]

30

*HuIL12 p40 subunit (This construct is a cDNA of the p40 mRNA. The DNA coding for its native signal peptide is in italics, and this is followed by DNA coding for the mature p40):*

35 **ATGTGTCACCAGCAGTTGGTCATCTCTTGGTTTTCCCTGGTTTTTCTGGC**  
**ATCTCCCCCTCGTGGCCATATGGGAACTGAAGAAAGATGTTTATGTGC**  
 TAGAATTGGATTGGTATCCGGATGCCCTGGAGAAATGGTGGTCCT  
 CACCTGTGACACCCCTGAAGAAGATGGTATCACCTGGACCTTGGAC  
 CAGAGCAGTGAGGTCTTAGGCTCTGGCAAAACCCTGACCATCCAAG  
 40 TCAAAGAGTTTGGAGATGCTGGCCAGTACACCTGTCACAAAGGAGG  
 CGAGGTTCTAAGCCATTCGCTCCTGCTGCTTCACAAAAAGGAAGAT  
 GGAATTTGGTCCACTGATATTTTAAAGGACCAGAAAGAACCCAAAA  
 ATAAGACCTTTCTAAGATGCGAGGCCAAGAATTATTCTGGACGTTTC

ACCTGCTGGTGGCTGACGACAATCAGTACTGATTTGACATTCAGTGT  
CAAAAGCAGCAGAGGGCTCTTCTGACCCCCAAGGGGTGACGTGCGGA  
GCTGCTACACTCTCTGCAGAGAGAGTCAGAGGGGACAACAAGGAGT  
ATGAGTACTCAGTGGAGTGCCAGGAGGACAGTGCCTGCCAGCTGC  
5 TGAGGAGAGTCTGCCCATTGAGGTCATGGTGGATGCCGTTCAACAAG  
CTCAAGTATGAAAACCTACACCAGCAGCTTCTTCATCAGGGACATCA  
TCAAACCTGACCCACCCAAGAACTTGCAGCTGAAGCCATTAAAGAA  
TTCTCGGCAGGTGGAGGTCAGCTGGGAGTACCCTGACACCTGGAGT  
ACTCCACATTCTACTTCTCCCTGACATTCTGCGTTCAGGTCCAGGG  
10 CAAGAGCAAGAGAGAAAAGAAAGATAGAGTCTTCACGGACAAGAC  
CTCAGCCACGGTCATCTGCCGCAAAAATGCCAGCATTAGCGTGCGG  
GCCCAGGACCGCTACTATAGCTCATCTTGGAGCGAATGGGCATCTG  
TGCCCTGCAGTTAG

15 [SEQ ID NO:10]

Alternatively, the nucleic acid molecule comprises nucleotide sequences that are degenerate sequences of those nucleotide sequences identified above (*i.e.* which encode the same amino acid sequence).

20

Preferably, the nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 8.

Advantageously, the nucleic acid molecule comprises the nucleotide sequence of  
25 SEQ ID NO: 9.

Conveniently, the nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 8 and the nucleotide sequence of SEQ ID NO: 9.

30 Suitably, the nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 10.

A further aspect of the present invention provides a method of making a compound according to the first aspect of the invention, said method comprising  
35 expressing one or more nucleic acid molecules according to the second aspect of the invention in a host cell and isolating the compound therefrom (see Example 1).

It is preferable that the two portions of the compound of the invention are produced as a fusion compound by recombinant DNA techniques, whereby a length of DNA comprises respective regions encoding the two portions of the compound of the invention either adjacent one another or separated by a region  
5 encoding a linker peptide which does not destroy the desired properties of the compound.

The nucleic acid may be expressed in a suitable host to produce a polypeptide comprising the compound of the invention. Thus, the nucleic acid encoding the  
10 compound of the invention or a portion thereof may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the compound of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859  
15 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to  
20 Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

Where the compound of the invention is multimeric, the constituent chains may be encoded by a single nucleic acid molecule or separate nucleic acid molecule  
25 (expressed in a common host cell or in different host cells and assembled *in vitro*).

The nucleic acid encoding the compound of the invention or a portion thereof may be joined to a wide variety of other nucleic acid sequences for introduction into an appropriate host. The companion nucleic acid will depend upon the nature of the  
30 host, the manner of the introduction of the nucleic acid into the host, and whether episomal maintenance or integration is desired.

It will be appreciated that in order to prevent expression of the cytotoxic portion of the compound of the invention from killing the host cells in which it is expressed, it may be necessary to link the nucleic acid of the second aspect of the invention to a signal sequence capable of directing secretion of the expressed compound (or  
5 portion) out of the host cell. Signal sequences will be selected according to the type of host cell used. Exemplary signal sequences include the *ompA* signal sequence (for example, see Takahara *et al.*, 1985, *J. Biol. Chem.* **260**(5):2670-2674).

10 Generally, the nucleic acid is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the nucleic acid may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. For example, the  
15 nucleic acid molecule encoding a compound of the invention may be linked to or comprise a Kozak consensus ribosome binding sequence (such as GCCGCCACC) to enhance translation.

The vector is then introduced into the host through standard techniques.  
20 Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a nucleic acid sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can  
25 be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant nucleic acid of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to  
30 permit the expression of the polypeptide, which can then be recovered.

Many expression systems are known, including bacteria (for example *E. coli* and

*Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae* and *Pichia pastoris*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells (for example COS-1, COS-7, CHO, NIH 3T3, NS0 and BHK cells) and insect cells (for example *Drosophila*, SF9 cells).

5

Those vectors that include a replicon such as a procaryotic replicon can also include an appropriate promoter such as a procaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

10

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

15

Typical procaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 (available from Biorad Laboratories, Richmond, CA, USA), p*Trc*99A and pKK223-3 (available from Pharmacia Piscataway, NJ, USA) and the pET system (T7 promoter, Novagen Ltd).

20

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

25

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

30

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally

available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *his3*, *trp1*, *leu2* and *ura3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

5

Further useful vectors for transformation of yeast cells, such as *Pichia*, include the 2 $\mu$  plasmid pYX243 (available from R and D Systems Limited) and the integrating vector pPICZ series (available from Invitrogen).

10 A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

15

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that  
20 remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess  
25 of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been  
30 cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

- 5 A desirable way to modify the nucleic acid encoding the compound of the invention or a portion thereof is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491.

10 In this method the nucleic acid to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified nucleic acid. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

- 15 Exemplary genera of yeast contemplated to be useful in the practice of the present invention are *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Hansenula*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodospiridium*, *Leucosporidium*, *Botryosascus*, *Sporidiobolus*, *Endomycopsis*, and the like. Preferred genera are those selected from the group
- 20 consisting of *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Yarrowia* and *Hansenula*. Examples of *Saccharomyces* are *Saccharomyces cerevisiae*, *Saccharomyces italicus* and *Saccharomyces rouxii*. Examples of *Kluyveromyces* are *Kluyveromyces fragilis* and *Kluyveromyces lactis*. Examples of *Hansenula* are *Hansenula polymorpha*, *Hansenula anomala* and *Hansenula capsulata*. *Yarrowia*
- 25 *lipolytica* is an example of a suitable *Yarrowia* species.

Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference.

30

Suitable promoters for *S. cerevisiae* include those associated with the *PGK1* gene, *GAL1* or *GAL10* genes, *CYC1*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes for

glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase,  $\alpha$ -mating factor pheromone, a-mating factor pheromone, the *PRB1* promoter, the *GUT2* promoter, and hybrid promoters involving hybrids of parts of  
5 5' regulatory regions with parts of 5' regulatory regions of other promoters or with upstream activation sites (e.g. the promoter of EP-A-258 067).

The transcription termination signal is preferably the 3' flanking sequence of a eukaryotic gene which contains proper signals for transcription termination and  
10 polyadenylation. Suitable 3' flanking sequences may, for example, be those of the gene naturally linked to the expression control sequence used, i.e. may correspond to the promoter. Alternatively, they may be different in which case the termination signal of the *S. cerevisiae AHD1* gene is preferred.

15 The present invention also relates to a host cell transformed with a polynucleotide vector construct of the present invention. The host cell can be either procaryotic or eukaryotic. Bacterial cells are preferred procaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available  
20 from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Preferred eukaryotic host cells include NS0 cells, Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse  
25 embryo cells NIH/3T3 available from the ATCC as CRL 1658 and monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650 or WSØ cells.

Transformation of appropriate cell hosts with a nucleic acid constructs of the present invention is accomplished by well known methods that typically depend  
30 on the type of vector used. With regard to transformation of procaryotic host cells, see, for example, Cohen *et al*, *Proc. Natl. Acad. Sci. USA*, **69**: 2110 (1972); and Sambrook *et al*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor

Laboratory, Cold Spring Harbor, NY (1989). Transformation of yeast cells is described in Sherman *et al*, *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY (1986). The method of Beggs, *Nature*, **275**: 104-109 (1978) is also useful. With regard to vertebrate cells, reagents useful in  
5 transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc, Gaithersburg, MD 20877, USA.

Successfully transformed cells, *i.e.* cells that contain a nucleic acid construct of the  
10 present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct of the present invention can be grown to produce the polypeptide of the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern, *J. Mol. Biol.*, **98**: 503 (1975) or  
15 Berent *et al*, *Biotech.*, **3**: 208 (1985). Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

In addition to directly assaying for the presence of recombinant nucleic acid, successful transformation can be confirmed by well known immunological  
20 methods when the recombinant nucleic acid is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

25 Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium. Preferably, the culture also contains the protein.

30 Nutrient media useful for culturing transformed host cells are well known in the art and can be obtained from several commercial sources.

A third aspect of the invention provides a vector comprising a nucleic acid according to the second aspect of the invention.

- 5 A fourth aspect of the invention provides a host cell comprising a vector according to the third aspect of the invention.

Preferably, the host cell is a mammalian cell, such as an NS/0 or CHO cell.

- 10 The compound of the invention may be purified from the culture medium using, in sequence, some or all of the following steps: Abx Mixed Resin column chromatography, recombinant Protein A chromatography, and Q Sepharose column chromatography, followed by Pellicon 2 tangential flow diafiltration for buffer exchange into formulation buffer. Virus inactivation and removal steps  
15 may be interdigitated into these steps. The virus inactivation and removal steps are not necessary for purification *per se*, but are used to satisfy regulatory considerations

- Detailed methods suitable for producing compounds of the invention are described  
20 in Gillies *et al.* (WO 99/29732, incorporated herein by reference). Other suitable techniques are described in *Molecular Cloning: a Laboratory Manual: 3rd edition*, Sambrook and Russell, 2001, Cold Spring Harbor Laboratory Press.

- A fifth aspect of the invention provides a pharmaceutical composition comprising  
25 a compound according to the first aspect of the invention and a pharmaceutically acceptable carrier.

- Preferably, the compound, *e.g.* fusion protein, may be formulated in phosphate buffered saline (PBS), in buffers containing arginine, citrate, mannitol, and/or  
30 Tween, or other standard protein formulation agents.

Advantageously, the composition is suitable for parenteral administration.

Conveniently, the formulation is a unit dosage containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of the active compound.

- 5 The compounds of the invention will normally be administered orally or by any parenteral route, in the form of a pharmaceutical formulation comprising the active ingredient, optionally in the form of a non-toxic organic, or inorganic, acid, or base, addition salt, in a pharmaceutically acceptable dosage form. Depending upon the disorder and patient to be treated, as well as the route of administration, 10 the compositions may be administered at varying doses.

- In human therapy, the compounds of the invention can be administered alone but will generally be administered in admixture with a suitable pharmaceutical excipient diluent or carrier selected with regard to the intended route of 15 administration and standard pharmaceutical practice.

- For example, the compounds of the invention can be administered orally, buccally or sublingually in the form of tablets, capsules, ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, 20 delayed- or controlled-release applications. The compounds of invention may also be administered *via* intracavernosal injection.

- Such tablets may contain excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate and glycine, 25 disintegrants such as starch (preferably corn, potato or tapioca starch), sodium starch glycollate, croscarmellose sodium and certain complex silicates, and granulation binders such as polyvinylpyrrolidone, hydroxypropylmethylcellulose (HPMC), hydroxy-propylcellulose (HPC), sucrose, gelatin and acacia. Additionally, lubricating agents such as magnesium stearate, stearic acid, glyceryl 30 behenate and talc may be included.

Solid compositions of a similar type may also be employed as fillers in gelatin

capsules. Preferred excipients in this regard include lactose, starch, a cellulose, milk sugar or high molecular weight polyethylene glycols. For aqueous suspensions and/or elixirs, the compounds of the invention may be combined with various sweetening or flavouring agents, colouring matter or dyes, with  
5 emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof.

The compounds of the invention can also be administered parenterally, for example, intravenously, intra-arterially, intraperitoneally, intrathecally,  
10 intraventricularly, intrasternally, intracranially, intra-muscularly or subcutaneously, or they may be administered by infusion techniques. They are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH  
15 of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well-known to those skilled in the art.

Formulations suitable for parenteral administration include aqueous and non-  
20 aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and  
25 vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

30 The physician will determine the actual dosage which will be most suitable for any individual patient and it will vary with the age, weight and response of the particular patient. The dosages described in Example 9 are exemplary of the

average case. There can, of course, be individual instances where higher or lower dosage ranges are merited and such are within the scope of this invention.

5 A sixth aspect of the invention provides a compound according to the first aspect of the invention for use in medicine.

10 A seventh aspect of the invention provides the use of a compound according to the first aspect of the invention in the preparation of a medicament for treating a patient with cancer.

An eighth aspect of the invention provides a method of treating a patient with cancer, the method comprising administering a compound according to the first aspect of the invention to said patient.

15 In principle, the compounds and compositions of the invention may be used to treat any mammal, including pets such as dogs and cats and agriculturally important animals such as cows, horses, sheep and pigs.

20 Preferably, however, the patient is human.

The compounds of the invention are particularly suited to the treatment of solid tumours, such as glioblastomas. Other preferred indications include ovarian, gastric, colorectal and pancreatic cancers.

25 The invention will now be described in detail with reference to the following non-limiting examples:

30 Figure 1 shows a schematic diagram of preferred configurations of antibody variable regions (striped ovals), constant regions (white ovals), the IL-12 p35 subunit (small rectangles), the IL-12 p40 subunit (large rectangles), antibody hinges and linkers (thick lines) and disulfide bonds (thin lines). Particular preferred embodiments include intact IgG-type antibodies with p35 fused to the C-

terminus of the heavy chain and p40 attached to p35 by a disulfide bond (Figure 1A), a 'minibody' with the antibody V regions connected by a linker and attached through a hinge to a CH3 domain, and p35 fused to the C-terminus of the heavy chain and p40 attached to p35 by a disulfide bond (Figure 1B), an sFv with p35 fused to a V region and p40 attached by a disulfide bond (Figure 1C), and an Fab with p35 fused to a C region and p40 attached by a disulfide bond (Figure 1D). The IL-12 p35 subunit may also be attached to the N-terminus of a V region. The IL-12 p40 subunit may be attached to p35 through a disulfide bond or through a linker, yielding a so-called 'single-chain IL-12' moiety (scIL-12).

Figure 2 shows construct pdHL11-huBC1-M1-hup35 (see Example 1). The following features are depicted:

	<u>Nucleotide positions</u>	<u>Description</u>
15	1 (EcoRI) to 664 (XbaI)	CMV enhancer and promoter
	664 (XbaI) to 1114	genomic leader of a mouse immunoglobulin L chain
20	1115 to 1439	VL
	1440 (BamHI at 1447) to 1867	Intron between VL and CL
25	1868 to 2190	CL coding region and translation stop codon
	2191 to 3054 (SalI)	3' untranslated region and polyadenylation signal of the human immunoglobulin kappa chain gene
30	3054(SalI) to 3721 (XhoI)	CMV enhancer and promoter
	3721 (XhoI) to 4176	genomic leader of a mouse immunoglobulin L chain
35	4177 to 4534	VH
40	4535 (HindIII at 4542) to 6347	genomic sequence of human immunoglobulin $\gamma$ 1 gene constant region with deImmunised M1 at the fusion junction

	6348 to 6941	Hu p35 coding region and translation stop codon
5	6942 (XhoI at 6944) to 7190	3'-untranslated region and polyadenylation signal of SV40 late region
10	7191 to 9484 (EcoRI)	origin of replication and $\beta$ -lactamase gene from pBR322
	9484 (EcoRI) to 9713	Crippled SV40 enhancer and promoter
15	9714 to 10277	DHFR cDNA
	10278 to 10362	3'-untranslated region of DHFR fused to polyadenylation signal of SV40 early region via ligation of BglII sticky end to BclI sticky end
20	10363 to 10599	polyadenylation signal of SV40 early region

25 Figure 3 shows construct pNeo-CMV-hu p40 (see Example 1). The following features are depicted:

	<u>Nucleotide positions</u>	<u>Description</u>
30	218 to 871	CMV enhancer and promoter
	888 to 953	Native signal peptide of hu p40
35	954 to 1874	Hu p40 mature sequence and translation stop codon
	1884 to 2292	Murine kappa polyadenylation signal
40	2299 to 4591	Origin of replication and $\beta$ -lactamase gene from pBR322
	3526 to 4386	$\beta$ -lactamase gene
45	5630 to 6424	Neomycin-resistant gene

Figure 4 shows the binding of four constructs (huBC1-muIL12, huBC1-huIL12, muBC1 and huBC1) to recombinant oncofoetal fibronectin fragments FN789 and FN7B89 (see Example 3).

5 Figure 5A shows a titration of number of U-87MG cells injected versus rate of growth of subcutaneous tumours (see Example 5). Figure 5B shows the anti-tumour efficacy of huBC1-muIL12 in U87-MG subcutaneous model in SCID mice.

10 Figure 6 shows the anti-tumour efficacy of huBC1-muIL12 in A431 subcutaneous model in SCID mice (see Example 5).

Figure 7 shows the anti-tumour efficacy of huBC1-muIL12 in PC3mm2 subcutaneous model in SCID mice (see Example 5).

15

Figure 8 shows the anti-tumour efficacy of huBC1-muIL12 in HT-29 subcutaneous model in SCID mice (see Example 5).

20 Figure 9 shows the effect of huBC1-muIL12 administration on (A) lung surface covered by metastases and (B) lung weight human following injection of prostate carcinoma PC3mm2 cells into severe combined immunodeficient (SCID) mice (see Example 5).

25 Figure 10 shows a pharmacokinetic analysis of huBC1-muIL12 and huBC1-huIL12 in mice (see Example 5). BALB/c mice were injected with 25 mg of huBC1-IL12 in the tail vein. At various time points, small blood samples were taken by retro-orbital bleeding. The plasma was assayed by capture with anti-human IgG H&L antisera and detection with an anti-human or anti-murine IL12 antibody (R&D Systems). Results were normalised to the initial concentration in  
30 the serum of each mouse taken immediately after injection ( $t=0$ ). The circulating half-life in mice is about 19 hr for both molecules.

## EXAMPLES

### *Example 1 – Production of huBC1-huIL12 fusion protein*

#### 5 I. Construction of the expression vectors for huBC1-huIL12

##### *1. Variable region of the light chain (VL)*

10 The DNA encoding the variable region of the light chain (VL) of the humanised BC1 antibody was provided in the form of a plasmid, RKA.pMMR010. Polymerase chain reaction (PCR) was used to adapt the VL DNA for the expression vector pdHL11. The forward primer has the sequence 5'-C TTA AGC GAA ATT GTG TTG ACG CAG TC-3' [SEQ ID NO:11], where CTTAAG is an AflIII restriction site and GAA is the N-terminal amino acid residue of the mature  
15 VL. The reverse primer has the sequence 5'- GGATCCACTTACG TTT GAT CTC CAG CTT GG-3' [SEQ ID NO:12], where the underlined sequence hybridised to the 3' end of the VL and GGATCC adds a BamHI restriction site downstream of the VL splice donor site.

20 A genomic signal peptide sequence from a mouse immunoglobulin light chain gene was used for secretion of the light and heavy chains of huBC1 fusion protein. A Kozak consensus sequence CCACCATGG was introduced for optimal ribosome binding for translation initiation at ATG [Kozak (1984) *Nature* 308:241]. This was achieved by mutating the first amino acid residue after the  
25 translation initiation codon from AAG to GAG to give the sequence TCTAGACCCACCATGGAG [SEQ ID NO:13], where the Kozak consensus sequence is underlined and an XbaI restriction site (TCTAGA) is placed at the 5' end.

30 At the 3' end of the signal peptide, the gene sequence encoding the -2 amino acid residue (the -1 amino acid being the C-terminal residue of the signal peptide) was mutagenised from a serine residue to a leucine residue (AGC to TTA) so that the

DNA encoding the end of the signal peptide is *CTTAAGC*, where *CTTAAG* is a created AflII site [Lo *et al.* (1998) *Protein Engineering* **11**:495]. Therefore, the signal peptide sequence contains a substitution at the first amino acid residue after the initiation codon and another substitution at the amino acid residue at the -2 position. Since the signal peptide is cleaved off by signal peptidase inside the cell and does not appear in the secreted protein, these mutations do not affect the composition of the antibody product. The 450-bp XbaI-AflII fragment containing the genomic signal peptide sequence was ligated to the AflII-BamHI fragment encoding the VL to give an XbaI-BamHI fragment, and this was in turn inserted into the pdHL11 expression vector, which already contains transcription regulatory elements and immunoglobulin constant region sequences (see below).

## 2. *Variable region of the heavy chain (VH)*

The DNA encoding the variable region of the heavy chain (VH) of the humanised BC1 antibody was obtained in the form of a plasmid RHA.pGamma1. Polymerase chain reaction (PCR) was used to adapt the VH DNA for the expression vector pdHL11. The forward primer has the sequence 5'-*C TTA AGC GAG GTG CAG CTG GTG CAG TC*-3' [SEQ ID NO:14], where *CTTAAG* is an AflII restriction site and *GAG* is the N-terminal amino acid residue of the mature VH. The reverse primer has the sequence 5'-*AAGCTTACT TAC CTG AGG AGA CGG AGA CC*-3' [SEQ ID NO:15], where the underlined sequence hybridised to the 3' end of the VH and *AAGCTT* adds a HindIII restriction site downstream of the VH splice donor site.

25

Prior to ligation to the VH DNA, the XbaI site of the 450-bp XbaI-AflII fragment containing the genomic signal peptide sequence was converted to an XhoI site by linker ligation to give the sequence *CCTCGAGGCTAGACCA~~CCAT~~GGAG* [SEQ ID NO:16], where *CCTCGAGG* is the sequence of the XhoI linker, CTAGA is the XbaI sticky end made blunt by filling in with the Klenow fragment of DNA polymerase, and *CCACCA~~TT~~GG* is the Kozak consensus sequence. The XhoI-AflII restriction fragment containing the genomic leader was ligated to the

30

AflIII-HindIII fragment containing the VH gene, and the resultant XhoI-HindIII fragment was then inserted into the pdHL11 expression vector, which already contains transcription regulatory elements and immunoglobulin constant region sequences (see below).

5

### 3. *Human constant regions*

The light chain construct uses the constant region of the human kappa chain gene and the heavy chain construct uses the constant regions of the human gamma-1  
10 chain. There is a SmaI restriction site located 280 bp upstream of the translation stop codon in the wild-type DNA sequence encoding the CH3 domain. This SmaI site was destroyed by the introduction of a silent mutation (TCC to TCA). Another silent mutation was introduced 10 bp upstream of the stop codon to create the sequence C CCG GGT AAA (STOP) [SEQ ID NO:17], which contains a new  
15 SmaI site [Lo *et al.* (1998) *Protein Engineering* **11**:495]. This SmaI site is now unique in the pdHL11 expression vector and is used for as a fusion junction for creating antibody-cytokine fusion proteins.

### 4. *cDNAs encoding the p35 and p40 subunits of human IL-12*

20

The cDNAs of p35 and p40 subunit of human IL12 were cloned from human peripheral blood monocytes (PBMC) using polymerase chain reactions (PCR). First strand cDNA was synthesised using an oligo dT primer and reverse transcriptase. The cDNA product was used as template for PCR. For the p35  
25 subunit, the sense primer has the sequence 5'-CCAGAAAGCAAGA GACCAGAG-3' [SEQ ID NO:18], and the antisense primer has the sequence 5'-GGAGGGACCTCGAGTTTTAGGAAGCATTTCAG-3' [SEQ ID NO:19]. The sense primer is derived from a sequence in the 5' untranslated region of the p35 message just upstream of a XmaI site, while the antisense primer encodes a  
30 translational stop codon followed shortly thereafter by a XhoI site for directional cloning. The primers for the p40 subunit cDNA were 5'-CTCCGTCCTGTCT AGAGCAAGATGTGTC-3' [SEQ ID NO:20] for the sense and

5'-GCTTCTCGAGAACCTAACTGCAGGGCACAG-3' [SEQ ID NO:21] for the antisense primer. The sense primer adds a unique XbaI site upstream of the translation start site while the antisense primer adds a XhoI site downstream of the translation stop codon.

5

#### 5. Construction of huBC1-H chain-human p35 DNA

The cloned p35 cDNA, after sequence confirmation, was adapted for expression as a fusion protein as follows. At the fusion junction, the C-terminal amino acid residue of the CH3 is lysine and the N-terminal residue of the mature p35 is arginine. To minimise proteolysis at the fusion junction with these two basic residues, both of them were mutagenised to alanine, which, in the case of IL2 immunocytokine, has been shown to extend serum half-life [Gillies *et al.* (2002) *Clin. Cancer Res.* 8:210]. For reconstruction of the fusion junction, there is a convenient BalI site just 11 base-pairs (bp) downstream of the mature N-terminus of p35. Hence a XmaI-BalI oligonucleotide linker consisting of sense strand 5'-CCG GGC **GCC GCA** AAC CTC CCC GTG G-3' [SEQ ID NO:22] and anti-sense strand 5'-C CAC GGG GAG GTT **TGC GGC** GC-3' [SEQ ID NO:23], where the **GCC GCA** denote the two alanine substitutions, was synthesised and ligated to a BalI-XhoI restriction fragment encoding the rest of the p35 subunit. The resultant XmaI-XhoI fragment in turn was ligated to the unique XmaI site in the pdHL11 expression vector, forming the CH3-p35 fusion junction. The peptide sequence at the junction, LSLSPGAANLPV [SEQ ID NO:24], where **AA** are the two alanine substitutions, is novel and potentially immunogenic. Indeed it contained a potential T helper cell epitope, which could be removed by mutating the LSLS residues to ATAT, based on Biovation's technology called deImmunization. The resultant deImmunised fusion junction sequence is called M1. Therefore, the huBC1-H chain-M1-hu p35 DNA consists of the XhoI-HindIII fragment encoding the signal peptide-VH, the HindIII-XmaI fragment encoding the genomic human IgG1 H chain constant regions with the deImmunised junction, and the XmaI-XhoI fragment encoding the p35 subunit.

6. *Expression vector pdHL11-huBC1-hu p35*

The expression vector pdHL11 is derived from pdHL7, which had been described previously (Gillies *et al.* (1998) *J. Immunol.* **160**:6195). As in pdHL7, the two  
5 transcriptional units for the L and H chains in pdHL11 contain the CMV enhancer-promoter [Boshart *et al.* (1985) *Cell* **41**:521-530]. The DNA for the CMV enhancer-promoter was obtained from an AflIII-HindIII fragment of the commercially available pcDNAI (Invitrogen Corp., San Diego, CA). At the 3' end, the L chain uses the 3' untranslated region and polyadenylation signal of the  
10 human immunoglobulin kappa chain gene and the H chain uses the 3'-untranslated region and polyadenylation signal of the SV40 late region.

The major difference between pdHL7 and pdHL11 is in the transcription unit for the dihydrofolate reductase (DHFR) selection marker. The SV40 enhancer for this  
15 transcription unit was destroyed in pdHL11 as follows. There are two 72 bp repeats in the SV40 enhancer/promoter, and within each 72 bp is a SphI restriction site. Ligation of the SalI site 5' of the enhancer to the distal SphI site through an oligonucleotide linker-adaptor resulted in the deletion of 120 bp from the two 72 bp repeats. Such an enhancerless promoter should give a much lower expression  
20 level of the DHFR selection marker. This, in theory, should result in fewer stably transfected cell clones, which, in order to survive the drug selection, might have the plasmid integrated into an active transcription region of a chromosome so that sufficient DHFR was expressed from the enhancerless promoter. The genes of interest, driven by fully functional enhancers and promoters, should be expressed  
25 at even higher levels in this active transcription region. In addition, the orientation of this attenuated transcription unit was reversed in pdHL11, so that the CMV enhancer for the L chain cannot exert a direct effect on the distal SV40 promoter for the expression of DHFR.

30 The construct pdHL11-huBC1-M1-hup35 was extensively mapped by restriction endonuclease digestions. The coding regions of the entire L and H chains were completely sequenced. Its salient features are depicted in Fig. 2.

### 7. Expression vector for the hu p40 subunit

The cloned p40 cDNA containing the complete open reading frame, after sequence  
 5 confirmation, was ligated into a separate expression vector as an XbaI-XhoI  
 fragment. This expression vector, pNeo-CMV-hu p40, contains a neomycin  
 resistance gene for selection of transfected cells using the neomycin analog G418.  
 The expression of the p40 is under the control of the CMV enhancer-promoter,  
 and utilises the murine kappa polyadenylation signal.

10 The construct pNeo-CMV-hu p40 was extensively mapped by restriction  
 endonuclease digestions. Its salient features are depicted in Figure 3.

## 15 II. DNA and protein sequences of huBC1-huIL12

### 1. Peptide and DNA sequence of the light chain of huBC1-huIL12

The peptide sequence of the secreted light chain of the humanised BC1-huIL12  
 20 is as follows (VL is underlined):

EIVLTQSPGTLSLSPGERATLSCSASSSISSNYLHWYQOKPGQAPRLLIYR  
TSNLASGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCOQGSSIPFTFGQG  
TKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV  
 25 DNALQSGNSQESVTEQDSKDYSLSSLTLSKADYEKHKVYACEVTH  
 QGLSSPVTKSFNRGEC

[SEQ ID NO:7]

The DNA sequences of the light chain construct, starting from the translation  
 30 initiation codon **ATG** to the stop codon **TAG**, is given below. (VL is underlined;  
 the upper and lower cases represent the coding and non-coding sequences,  
 respectively):

**ATG**GAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCCTGgt  
 35 gaggagagaggggaagtgagggaggagaatggacagggagcaggagcactgaatccattgctcattccatgta  
 tctggcatgggtgagaagatgggtcttatctccagcatggggcctctggggtgaatactgttagagggaggttc  
 cagatgggaacatgtgtataatgaagattatgaaatggatgcctgggatgggtctaagtaatgccttagaagtgact

agacacttgcaattcacttttttggaagaagagatttttaggcataaaaaaatgttatgtaaaaaataaacgatcaca  
 gttgaaataaaaaaaaaataaaggatgttcataaattttgtgtataactatgtatttctctctcattgttcagCTTC  
 CTTAAGCGAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGT  
 CTCCAGGGGAAAGAGCCACCCTCTCCTGCAGTGCCAGTTCAAGTAT  
 5 AAGTTCCAATTACTTGCATTGGTACCAGCAGAAACCTGGCCAGGCT  
CCCAGGCTCCTCATCTATAGGACGTCCAATCTGGCTTCTGGCATCCC  
AGACAGGTTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACC  
ATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGC  
AGGGTAGTAGTATACCATTACGTTTGGCCAGGGGACCAAGCTGGA  
 10 GATCAAACgtaagtggatcctatcagggtttacaagagggactaaagacatgtcagctatgtgtgactaat  
 ggtaatgtcactaagctgcgcgatcccgaattctaaactctgagggggcggatgacgtggccattctttgcctaa  
 agcattgagtttactgcaaggtcagaaaagcatgcaaagccctcagaatggctgcaaagagctccaacaaacaa  
 tttagaactttattaaggaatagggggaagctaggaagaaactcaaaacatcaagattttaaatacgtcttctgtctc  
 cttgtataattatctgggataagcatgctgtttctgtctgtccctaacatgccctgtgattatccgcaaacaacacac  
 15 ccaagggcagaaactttgttacttaaacaccatcctgtttgtcttctcagGAAGTGTGGCTGCACC  
 ATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAA  
 CTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCC  
 AAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCC  
 AGGAGAGTGTACAGAGCAGGACAGCAAGGACAGCACCTACAGCC  
 20 TCAGCAGCACCTGACGCTGAGCAAAGCAGACTACGAGAAACACA  
 AAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGT  
 CACAAAGAGCTTCAACAGGGGAGAGTGTTAG

[SEQ ID NO:9]

25

## 2. Peptide and DNA sequence of the heavy chain of huBC1-huIL12

The peptide sequence of the secreted heavy chain huBC1-hup35 is as follows (VH is underlined, the deImmunised M1 junction in italics, and human p35 in bold):

30

EVQLVQSGADVKKPGASVKVSCKASGYTFTNYVMHWVROAPGOGL  
WLGYNINPYNDGTQYNERFKGRVTMTGDTSSISTAYMELSRLTSDDTAVY  
YCAREVYGNYIWGNWGQGTLSVSVSSASTKGPSVFPLAPSSKSTSGGTA  
 ALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP  
 35 SSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGP  
 SVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHN  
 AKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE  
 KTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE  
 SNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE  
 40 ALHNHYTQKSATATPGAA~~NLPVATPD~~PGMFPCLHHSQNLLRAVSNM  
**LQKARQ**TLEFY**PCT**SEEIDHEDITKD**K**STVEAC**LPLELTKNESCLN**  
**SRET**SFITNGSCLAS**RKTSFMMALCLSSIYEDLKMYQVEFKTMNAK**  
**LLMDPKRQIFLDQ**NMLAVIDELMQALN**FNSETVPQKSSLEEPDFYK**  
**TKIKLCILLHA**FRIRAVTIDRVMSYLNAS

45

[SEQ ID NO:6]

The DNA sequences of the heavy chain huBC1-hu p35 construct, starting from the translation initiation codon **ATG** to the stop codon **TAA**, is given below (VH is underlined; the p35 sequence is in bold; and the upper and lower cases represent the coding and non-coding sequences, respectively):

ATGGAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCTGgt  
 gaggagagaggggaagtgagggaggagaatggacagggagcaggagcactgaatcccattgctcattccatgta  
 tctggcatgggtgagaagatgggtcttatcctccagcatggggcctctggggtgaataacttgttagaggagggtc  
 10 cagatgggaacatgtgctataatgaagattatgaaatggatgcctgggatgggtctaagtaatgccttagaagtgact  
 agacacttgcaattcacttttttgtaagaagagatttttaggctataaaaaaatgttatgtaaaaaataaacgatcaca  
 gttgaaataaaaaaaaaataaaggatgttcatgaattttgtgtataactatgtatttctctcattgtttcagCTTC  
 CTTAAGCGAGGTGCAGCTGGTGCAGTCTGGGGCTGACGTGAAGAAG  
 CCTGGGGCCTCAGTGAAGGTCTCCTGCAAGGCTTCTGGATACACCTT  
 15 CACCAACTACGTAATGCACTGGGTGCGACAGGCCCTGGACAAGGG  
 CTTGAGTGGCTGGGATATATTAATCCTTACAATGATGGTACTCAGTA  
 CAATGAGAGGTTCAAAGGCAGGGTCACCATGACCGGGGACACGTCC  
 ATCAGTACAGCCTATATGGAGCTGAGCAGGCTGACTTCTGACGACA  
 CCGCGGTGTATTACTGTGCGAGAGAGGTCTATGGTAACTACATCTG  
 20 GGGCAACTGGGGCCAGGGAACCCTGGTCTCCGTCTCCTCAGgtaagtaag  
 ctttctggggcaggccaggcctgacctggccttggggcagggagggggctaaggtgaggcaggtggcgccag  
 ccaggtgcacaccaatgccatgagcccagacactggacgctgaacctgcgagacgttaagaaccaggggg  
 cctctgcgccctggggccagctctgtcccacaccgcggtcacatggcaccacctctcttgcagCCTCCACC  
 AAGGGCCCATCGGTCTTCCCCCTGGCACCCCTCCTCCAAGAGCACCTC  
 25 TGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCC  
 GAACCGGTGACGGTGTCTGTGAACTCAGGCGCCCTGACCAGCGGCG  
 TGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTC  
 AGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCT  
 ACATCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAA  
 30 GAGAGTTGgtgagaggccagcacagggagggaggggtgtctgtggaagccaggctcagcgtcctgc  
 ctggacgcacccggctatgcagtcccagtcaggggcagcaaggcagggccccgtctgctcttccaccggagg  
 cctctgccccccccactcatgctcagggagaggggtcttctggccttttccccaggctctgggcaggccacaggctag  
 gtccccctaaccaggccctgcacacaaaggggcaggtgtctgggctcagacctgccaagagccatatccggga  
 ggacctgcccctgacctagcccaccccaaaggccaaactctccactccctcagctcggacaccttctctctcc  
 35 cagattccagtaactcccaatcttctctgcagAGCCCAAATCTTGTGACAAAATCAC  
 ACATGCCCAACCGTGCCCAgtaagccagccaggcctcgcctccagctcaaggcgggaca  
 ggtgccctagagtagcctgcatccaggacagggcccccagccgggtgtgacacgtccacctccatctcttctca  
 gCACCTGAACTCCTGGGGGGACCGTCAGTCTTCTTCTTCCCCCCAAA  
 ACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCAAGTTCAACT  
 40 GTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACT  
 GGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGC  
 GGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCAC  
 CGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAG  
 GTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCA  
 45 AAGCCAAAGgtgggacccgtgggggtgcgagggccacatggacagaggccggctcggccaccctct

gccctgagagtgaccgctgtaccaacctctgtccctacagGGCAGCCCCGAGAACCACAGG  
 TGTACACCCTGCCCCCATCACGGGAGGAGATGACCAAGAACCAGGT  
 CAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCC  
 GTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACCTACAAGACC  
 5 ACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCTCTATAGCAA  
 GCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCA  
 TGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGA  
 GCGCCACCGCGACCCCGGGCGCCGCAAACCTCCCCGTGGCCACTC  
 CAGACCCAGGAATGTTCCCATGCCTTCACCACTCCCCAAAACCTG  
 10 CTGAGGGCCGTCAGCAACATGCTCCAGAAGGCCAGACAAACTC  
 TAGAATTTTACCCTTGCACCTTCTGAAGAGATTGATCATGAAGAT  
 ATCACAAAAGATAAAAACCAGCACAGTGGAGGCCTGTTTACCATT  
 GGAATTAACCAAGAATGAGAGTTGCCTAAATTCCAGAGAGACCT  
 CTTTCATAACTAATGGGAGTTGCCTGGCCTCCAGAAAGACCTCT  
 15 TTTATGATGGCCCTGTGCCTTAGTAGTATTTATGAAGACTTGAA  
 GATGTACCAGGTGGAGTTCAAGACCATGAATGCAAAGCTTCTGA  
 TGGATCCTAAGAGGCAGATCTTCTAGATCAAAACATGCTGGCA  
 GTTATTGATGAGCTGATGCAGGCCCTGAATTTCAACAGTGAGAC  
 TGTGCCACAAAAATCCTCCCTTGAAGAACCGGATTTTTATAAAA  
 20 CTAATAATCAAGCTCTGCATACTTCTTCATGCTTTCAGAATTTCGG  
 GCAGTGACTATTGACAGAGTGACGAGCTATCTGAATGCTTCCTA  
 A

[SEQ ID NO:8]

25

### 3. Peptide and DNA sequence of the p40 subunit

The peptide sequence of the secreted human p40 subunit is as follows:

30 IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVL  
 GSGKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILK  
 DQKEPKNKTFLRCEAKNYSGRFTCWWLTISTDLTFSVKSSRGSSDPQG  
 VTCGAATLSAERVRGDNKEYEYSVECQEDSACPAAEESLPIEVMVDAV  
 HKLKYENYTSSFFIRDIIKPDPPKNLQLKPLKNSRQVEVSWEYPDTWSTP  
 35 HSYFSLTFCVQVQGKSKREKKDRVFTDKTSATVICRKNASISVRAQDR  
 YYSSSWSEWASVPCS

[SEQ ID NO:4]

40 The DNA sequences of the p40 construct, starting from the translation initiation  
 codon **ATG** to the stop codon **TAG**, is given below (The construct is a cDNA of  
 the p40 mRNA. The DNA coding for its native signal peptide is in italics, and this  
 is followed by DNA coding for the mature p40):

ATGTGTCACCAGCAGTTGGTCATCTCTTGGTTTTCCCTGGTTTTCTGGC  
ATCTCCCCTCGTGGCCATATGGGAACTGAAGAAAGATGTTTATGTCG  
TAGAATTGGATTGGTATCCGGATGCCCCCTGGAGAAATGGTGGTCCT  
CACCTGTGACACCCCTGAAGAAGATGGTATCACCTGGACCTTGGAC  
5 CAGAGCAGTGAGGTCTTAGGCTCTGGCAAAACCCTGACCATCCAAG  
TCAAAGAGTTTGGAGATGCTGGCCAGTACACCTGTCACAAAGGAGG  
CGAGGTTCTAAGCCATTTCGCTCCTGCTGCTTCACAAAAAGGAAGAT  
GGAATTTGGTCCACTGATATTTTAAAGGACCAGAAAGAACCCAAAA  
ATAAGACCTTTCTAAGATGCGAGGCCAAGAATTATTCTGGACGTTTC  
10 ACCTGCTGGTGGCTGACGACAATCAGTACTGATTTGACATTCAGTGT  
CAAAAGCAGCAGAGGGCTCTTCTGACCCCCAAGGGGTGACGTGCGGA  
GCTGCTACACTCTCTGCAGAGAGAGTCAGAGGGGACAACAAGGAGT  
ATGAGTACTCAGTGGAGTGCCAGGAGGACAGTGCCTGCCCAGCTGC  
TGAGGAGAGTCTGCCCATTTAGGTTCATGGTGGATGCCGTTTACAAG  
15 CTCAAGTATGAAAACCTACACCAGCAGCTTCTTCATCAGGGACATCA  
TCAAACCTGACCCACCCAAGAACTTGCAGCTGAAGCCATTAAAGAA  
TTCTCGGCAGGTGGAGGTCAGCTGGGAGTACCCTGACACCTGGAGT  
ACTCCACATTCCTACTTCTCCCTGACATTCTGCGTTCAGGTCCAGGG  
CAAGAGCAAGAGAGAAAAAGAAAGATAGAGTCTTCACGGACAAGAC  
20 CTCAGCCACGGTCATCTGCCGCAAAAATGCCAGCATTAGCGTGCGG  
GCCCAGGACCGCTACTATAGCTCATCTTGGAGCGAATGGGCATCTG  
TGCCCTGCAGTTAG

[SEQ ID NO:10]

25

*Example 2 – Binding characterisation (Study I)*

Surface plasmon resonance and immunostaining experiments were performed to characterise the binding of an exemplary BC1-IL12 fusion protein of the invention  
30 to oncofoetal fibronectin.

In the course of characterising the binding of the BC1-IL12 fusion protein to its target antigen, it was found that this fusion protein bound more tightly to its target than did the corresponding BC1 antibody itself. For example, the binding of BC1  
35 and BC1-IL12 to a polypeptide including human fibronectin domains 7, ED-B, 8, and 9 was measured using surface plasmon resonance. Table 1 summarises the results of two experiments.

40

**Table 1**

	muBC1 (murine constant regions)	HuBC1 (human constant regions)	huBC1-IL12
On-rate (1/mole/sec)	1.65x10 <sup>3</sup> (exp. 2)	6.2x10 <sup>4</sup> (exp.1) 7.3x10 <sup>4</sup> (exp.2)	1.7x10 <sup>4</sup> (exp.1) 1.9x10 <sup>4</sup> (exp.2)
Off-rate (1/sec)	1.1x10 <sup>-3</sup> (exp. 2)	7.8x10 <sup>-3</sup> (exp.1) 1.0x10 <sup>-2</sup> (exp.2)	1.3x10 <sup>-3</sup> (exp.1) 1.6x10 <sup>-3</sup> (exp.2)
Dissociation constant (nM)	686	125 (exp. 1) 138 (exp. 2)	7.6 (exp. 1) 8.3 (exp. 2)

- 5 The results indicate that the binding of huBC1-IL12 to its target antigen is at least 10-fold tighter, and most likely about 16-fold tighter, than the corresponding huBC1 antibody alone.

To confirm the results of the surface plasmon resonance study, U87 MG  
 10 subcutaneous tumours were generated in immuno-compromised SCID CB17 mice according to standard procedures, and tumour sections were immunostained with the huBC1 antibody and the huBC1-IL12 fusion protein. It was found that the intensity of staining with the huBC1-IL12 fusion protein was much greater than with the huBC1 antibody (data not shown).

15

### ***Example 3 – Binding characterisation (Study II)***

#### Introduction

- 20 Surface plasmon resonance (SPR) technology was used to demonstrate specificity of antigen binding (*i.e.* recognition of only the recombinant oncofoetal fibronectin, FN7B89) and to determine/compare the kinetic rate constants/affinity values for both murine and human BC1 antibodies and BC1-IL12 Immunocytokines. All

measurements reagents and software provided by Biocore (see appendix for list of reagents and software).

### Assay

5

ED-B negative (FN789) and ED-B positive (FN7B89) recombinant fibronectins (see 'Sequence information' below) were coupled on two different flow cells of a CM5 sensor chip using a standard amine coupling protocol and coupling reagents provided by Biacore. The other two flow cells were left blank and used a negative control surfaces. In order to demonstrate antigen specificity, the various BC1 antibodies and Immunocytokines were diluted to 500 nM in running buffer, HEPES Buffered Saline (HBS-EP). The samples were injected over the fibronectin-coupled surfaces for 5 min and the binding curves were compared. Running buffer (HBS-EP) was injected over each surface as a negative control to demonstrate baseline signal. The chip surfaces were regenerated with a 1 minute pulse of 0.1M HCl pH1.5 followed by a second 1 minute pulse of 0.1M H<sub>3</sub>PO<sub>4</sub>.

For kinetic analysis, only the ED-B positive fibronectin (FN7B89) was coupled to the chip. Three difference densities were coupled on three different flow cells. The fourth flow cell was left uncoupled and used as a negative control. Four to five concentrations of each molecule were prepared by performing twofold serial dilutions ranging from 1000nM to 125nM (muBC1), 200 nM to 25nM (huBC1) and 100nM to 6.25nM (murine and human BC1-IL12). The serial dilutions were made in triplicate in the running buffer (HBS-EP). Each dilution was injected for 5 min (association) followed by 5 min of running buffer (dissociation) at a flow rate of 10 TL/min. The flow cells were regenerated as was done in the antigen specificity experiments described above. Curve fitting was done using software provided by Biacore. See the appendix for specific details on curve fitting.

### 30 Results

While the various BC1 molecules bind with differing intensities to the

recombinant oncofoetal fibronectin, FN7B89, they do not bind at all to the recombinant “normal” fibronectin, FN789 (see Figure 4). This indicates that in all BC1 molecules tested, both antibodies and immunocytokines, have retained their antigen specificity (as compared to the original muBC1). These data also demonstrate that the kinetics of the antigen binding vary from molecule to molecule.

The kinetic analysis demonstrates that the rate constants do differ considerably between molecules (see Table 2).

**Table 2**

<b>Molecule</b>	<b>On-rate</b> $k_a$ ( $\times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ )	<b>Off-rate</b> $k_d$ ( $\times 10^{-3} \text{ s}^{-1}$ )	<b>Affinity</b> $K_D$ (nm)
muBC1	0.17	0.65	377
huBC1	5.76	3.12	54
HuBC1-muIL12	7.95	1.09	13.9
HuBC1-huIL12	5.09	0.87	17.3

Also, the BC1-IL12 immunocytokines have much higher affinity for FN7B89 than either the murine or human antibodies. Despite differences in the rate constants, huBC-1muIL12 and huBC1-huIL12 have essentially the same binding affinity for their antigen. These data indicate that the humanization of the BC1 antibody, as well as the subsequent generation of the BC1-IL12 immunocytokine, resulted in a molecule with increased affinity for the recombinant oncofoetal fibronectin.

### Conclusions

All of the BC1 molecules specifically bind to the recombinant oncofoetal fibronectin, FN7B89, indicating that the constructions of the hyBC-huIL12 immunocytokine has not resulted in the loss of antigen specificity. Humanization

of the BC1 murine antibody resulted in a molecule with increased binding affinity. This increase in the affinity is due to a significantly fast on-rate. The humanised antibody binds its antigen almost 34 times faster than its murine counterpart. However, humanization does have a negative impact as well. The off-rate for huBC1 is approximately 5 times faster than the muBC1. The addition of IL12 to the antibody, to create the BC1-IL12 immunocytokine, helps to off-set this, resulting in an off-rate similar to that seen from muBC1. *In vitro*, huBC1-huIL12 is a high affinity immunocytokine with the potential to be a potent tumour-targeting molecule *in vivo*.

10

### Sequence information

#### (a) *Fibronectin 789 fragment*

15 LOCUS FN789.DNA 1126 bp mRNA PRI 01-OCT-1999

DEFINITION Human mRNA for fibronectin domains 789 (no ED-B) in pQE12 (pAS32)

NID Derived from g31396 and pQE12 (Qiagen).

VERSION X02761.1 GI:31396

20 KEYWORDS alternate splicing; fibronectin.

SOURCE human.

ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;

Eutheria; Primates; Catarrhini; Hominidae; Homo.

25

CDS <208..1068

/product="Fn MRGS-789-HHHHHH"

/translation="

30

MRGSVVTPLSPPTNLHLEANPDTGVLTVSWERSTTPDI  
 TGYRITTTPTNGQQGNSLEEVVHADQSSCTFDNLSPGL  
 EYNVSVYTVKDDKESVPISDTIIPAVPPPTDLRFTNIG  
 PDTMRVTWAPPPSIDLTNFLVRYSPVKNEEDVAELIS  
 PSDNAVVLTNLLPGTEYVVS SVSSVYEQHESTPLRGRQK  
 TGLDSPTGIDFSDITANSFTVHWIAPRATITGYRIRHH

PEHFSGRPREDRVPHSRNSITLTNLTPGTEYVVSIVAL  
NGREESPLLIGRSRSHHHHHH"

[SEQ ID NO: 25]

5 Note1: Residue 1 to 207 is pQE sequence from and including Qiagen promoter primer (CCCGAAAAGTGCCACCTG). Residue 1069 to 1126 is pQE12 sequence from the end of the hexa-histidine tag to the Qiagen reverse primer sequence (GTTCTGAGGTCATTACTGG). Fibronectin-derived sequence (*i.e.* without MRGS and hexa-histidine tag is in lower case).

10

Note2: Please note that the coding sequence has mutations CC(230)A > CA(230)A leading to a P8Q change; A(286)CA > G(286)CA leading to a T27A change; and TCA(657) > TCG(657) leading to a silent S150S change.

15 BASE COUNT 319 a 297 c 226 g 284 t

ORIGIN

1 CCCCGAAAAG TGCCACCTGA CGTCTAAGAA ACCATTATTA  
TCATGACATT AACCTATAAA  
20 61 AATAGGCGTA TCACGAGGCC CTTTCGTCTT CACCTCGAGA  
AATCATAAAA AATTTAATTG  
121 CTTTGTGAGC GGATAACAAT TATAATAGAT TCAATTGTGA  
GCGGATAACA ATTTACACA  
181 GAATTCATTA AAGAGGAGAA ATTA ACTATG AGAGGATCtg  
25 tggtgacacc attgtctcca  
241 ccaacaaact tgcacttgga ggcaaacct gacactggag tgctcacagt ctctgggag  
301 aggagcacca cccagacat tactgggtat agaattacca caaccctac aaacggccag  
361 cagggaaatt ctttgaaga agtgggtccat gctgacaga gctcctgcac tttgataac  
421 ctgagtcctg gcctggagta caatgtcagt gttacactg tcaaggatga caaggaaagt  
30 481 gtccctatct ctgataccat catccagct gttcctctc cactgacct gcgattcacc  
541 aacattggtc cagacacat gcgtgtcacc tgggtccac cccatccat tgatttaacc  
601 aactcctgg tgcgttact acctgtgaaa aatgaggaag atgttcaga gttgtcaatt  
661 tctcctcag acaatgcagt ggtcttaaca aatctcctgc ctggtacaga atatgtagt  
721 agtgtctcca gtgtctacga acaacatgag agcacacct ttagaggaag acagaaaaca  
35 781 ggtcttgatt cccaactgg cattgacttt tctgatatta ctgccaactc ttttactgtg  
841 cactggattg ctctcgagc caccatcact ggctacagga tccgcatca tcccagcac  
901 ttcagtggga gacctgaga agatcgggtg cccactctc ggaattccat caccctcacc  
961 aacctcactc caggcacaga gtatgtggtc agcatcgttg ctctaatgg cagagaggaa  
1021 agtcccttat tgattggcaG ATCCAGATCT CATCACCATC  
40 ACCATCACTA AGCTTAATTA

1081 GCTGAGCTTG GACTCCTGTT GATAGATCCA GTAATGACCT  
CAGAAC

[SEQ ID NO: 26]

//

5

(b) *Fibronectin 7B89 fragment*

LOCUS FN7B89.DNA 1399 bp mRNA PRI 01-OCT-1999

DEFINITION Human mRNA for fibronectin domains 7B89 in pQE12 (pAS33)

10 NID Derived from g31396 and pQE12 (Qiagen).

VERSION X02761.1 GI:31396

KEYWORDS alternate splicing; fibronectin.

SOURCE human.

ORGANISM Homo sapiens

15 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;

Eutheria; Primates; Catarrhini; Hominidae; Homo.

CDS <208..1341

/product="Fn MRGS-7B89-HHHHHH"

/translation="

20 MRGSVVTPLSPPTNLHLEANPDTGVLTVSWERSTTPDI  
TGYRITTTPTNGQQGNSLEEVVHADQSSCTFDNLSPGL  
EYNVSVYTVKDDKESVPISDTIPEVPQLTDLSFVDIT  
DSSIGLRWTPPLNSSTIIGYRITVVAAGEGIPIFEDFVD  
SSVGYYTVTGLEPGIDYDISVITLINGGESAPTTLTQQ  
25 TAVPPPTDLRFTNIGPDTMRVTWAPPPSIDLTNFLVRY  
SPVKNEEDVAELSISPSDNAVVLTNLLPGTEYVVS  
VVEQHESTPLRGRQKTGLDSPTGIDFSDITANSFTVHW  
IAPRATITGYRIRHHPEHFSGRPREDRVPHSRNSITLT  
NLTPGTEYVVSIVALNGREESPLLIGRSRSHHHHHH"

30

[SEQ ID NO: 27]

Notel: Residue 1 to 207 is pQE sequence from and including Qiagen promoter  
primer (CCCGAAAAGTGCCACCTG). Residue 1342 to 1399 is pQE12 sequence  
from the end of the hexa-histidine tag to the Qiagen reverse primer sequence  
35 (GTTCTGAGGTCATTACTGG). Fibronectin-derived sequence (*i.e.* without  
MRGS and hexa-histidine tag is in lower case).

Note2: Please note that the coding sequence has mutations CC(230)A > CA(230)A leading to a P8Q change; A(286)CA > G(286)CA leading to a T27A change; and TCA(930) > TCG(930) leading to a silent S241S change.

5 BASE COUNT 390 a 368 c 290 g 351 t  
ORIGIN

```

1 CCCCCGAAAAG TGCCACCTGA CGTCTAAGAA ACCATTATTA
TCATGACATT AACCTATAAA
10 61 AATAGGCGTA TCACGAGGCC CTTTCGTCTT CACCTCGAGA
AATCATAAAA AATTTATTTG
121 CTTTGTGAGC GGATAACAAT TATAATAGAT TCAATTGTGA
GCGGATAACA ATTTACACA
181 GAATTCATTA AAGAGGAGAA ATTA ACTATG AGAGGATCtg
15 tggtgacacc attgtctcca
241 ccaacaaact tgcattctgga ggcaaaccct gacactggag tgctcacagt ctctggggag
301 aggagcacca cccagacat tactggttat agaattacca caaccctac aaacggccag
361 cagggaaatt ctttgaaga agtggtccat gctgatcaga gctcctgcac tttgataac
421 ctgagtcccg gcctggagta caatgtcagt gtttactg tcaaggatga caaggaaagt
20 481 gtccctatct ctgataccat catccagag gtgccccaac tctactgacct aagctttgtt
541 gatataaccg attcaagcat cggcctgagg tggacccgc taaactctc caccattatt
601 gggtagcgca tcacagtagt tgcggcagga gaaggtatcc ctattttga agattttgtg
661 gactcctcag taggatacta cacagtcaca gggctggagc cgggcattga ctatgatac
721 agcgttatca ctctcattaa tggcggcgag agtgccccta ctactgac acaacaacg
25 781 gctgttctc ctcccactga cctgcgatt accaacattg gtccagacac catgcgtgtc
841 acctgggctc caccctcacc cattgattta accaacttcc tggtagctta ctacctgtg
901 aaaaatgagg aagatgttgc agagtgttca atttctcct cagacaatgc agtggtctta
961 acaaatctcc tgcctgttac agaatatgta gtgagtgtct ccagtgtcta cgaacaacat
1021 gagagcacac ctcttagagg aagacagaaa acaggtcttg attccccaac tggcattgac
30 1081 tttctgata ttactgcaa ctcttttact gtgactgga ttgctctctg agccaccatc
1141 actggctaca ggatccgcca tcatcccgag cacttcagtg ggagacctcg agaagatcgg
1201 gtgccccact ctcggaattc catcacctc accaacctca ctccaggcac agagtatgtg
1261 gtcagcatcg ttgctcttaa tggcagagag gaaagtcct tattgattgg caGATCCAGA
1321 TCTCATCACC ATCACCATCA CTAAGCTTAA TTAGCTGAGC
35 TTGGACTCCT GTTGATAGAT
1381 CCAGTAATGA CCTCAGAAC

```

[SEQ ID NO: 28]

//

Materials and methods

1. Materials: Biacore AB, Uppsala  
catalog numbers and contact info available on website:  
5 [www.biacore.com](http://www.biacore.com)  
Biacore 2000  
BIAControl software (operates instrument)  
BIAEvaluation software (data analysis)  
Senior Chip CM5 (certified grade)  
10 HBS-EP  
Amine Coupling Kit
2. Kinetics Parameters: fit parameters selected in BIAEvaluation  
curve Fit = bivalent (analyte is the antibody)  
15 Start Injection = 0 sec  
Association = 30-270 secs (4 min)  
Stop Injection = 300 sec  
Dissociation = 330-600 (4.5 min)

*Example 4 – In vitro testing of efficacy in cancer therapy*

To verify the utility of BC1-IL12 fusion proteins in treatment of cancer, an  
 5 huBC1-muIL12 fusion protein was constructed and expressed according to  
 standard procedures (see Example 1 and Gillies *et al.*, WO99/29732, incorporated  
 herein by reference). This protein used murine IL-12 because human IL-12 is not  
 recognised by murine IL-12 receptors.

10 SCID CB17 mice bearing U87MG glioblastoma tumours with a volume of about  
 140 cubic millimetres were treated with either huBC1 or huBC1-IL12 as shown in  
 Table 3.

**Table 3**

15

Protein	Dose regimen	Tumour volume at day 8	Tumour volume at day 13
huBC1-IL12	20 mcg, day 0-7	85	60
huBC1-IL12	5 mcg, day 0-7	130	120
huBC1-IL12	5 mcg, day 0, 2, 4, 6, 10, 12	115	70
huBC1	400 mcg, day 0, 4	170	175
- (PBS)	Day 0-7	180	195

*Example 5 - Efficacy of huBC1-g1-muIL12 in Tumour Models in Mice*1. Introduction

5 The objective is to determine the efficacy of huBC1-IL12 in different tumour models in mice. HuBC1 (humanised BC1 antibody) targets the human fibronectin isoform, B-FN, that is present in the subendothelial extracellular matrix (ECM) of the neovasculature in vascularised tumours. B-FN is a good tumour marker because it is oncofoetal and angiogenesis-associated, and is undetectable in normal  
10 adult tissues. Since huBC-1 recognises only the human B-FN and does not cross-react with the murine B-FN, xenogeneic tumour models involving human tumour cells in severe combined immunodeficient (SCID) mice and nude mice were used for preclinical studies. Furthermore, since IL12 is species-specific, the huBC1-huIL12 (humanised BC1 antibody-human IL12 fusion protein) intended for  
15 humans does not work in mice. Therefore, we produced huBC1-murine IL12 as a surrogate drug candidate for evaluation in murine models.

2. Materials and methods20 *2.1 Mouse strains*

SCID CB17 and Nude mice were purchased from Taconic, Charles River, and Jackson Lab.

25 *2.2 Tumour cell lines*

The human prostate adenocarcinoma PC3mm2 was a gift from Dr. Ralph Reisfeld at Scripps Research Institute. The human astrocytoma U-87 MG, the human epidermoid carcinoma A431 and the human colon carcinoma HT29 were obtained  
30 from American Type Culture Collection.

### 2.3 Proteins

HuBC1-g1-muIL12 is the same as huBC1-g1-M1-muIL12. It is a fusion protein of the humanised BC1 antibody with the human g1 constant regions and murine IL-12, and M1 is a deImmunised fusion junction (see Example 1 above).

HuBC1-g1-muIL12 was produced in a similar manner as huBC1-huIL12 (see Example 1 above), except that the mup40 and mup35 replaced the hup40 and hup35, respectively.

10

### 3. Experimental design, dosing schedule and evaluation

#### *3.1 U-87MG Subcutaneous Model in SCID CB17 Mice*

15    02-23    Effect of HuBC1-g1-M1-muIL12 in Human U-87MG Astrocytoma Cells on Subcutaneous Model in SCID CB17 Mice

Mice:

7-week-old SCID CB17 mice, male

20

Tumour Injection:

Inject the subcutaneous dorsa of SCID CB17 mice in the proximal midline with  $4 \times 10^6$  viable U-87MG tumour cells in 0.1 ml PBS following the protocol.

25    Groups and treatment:

Treatment starts when tumour size reaches  $\sim 100\text{mm}^3$ . Mice are sorted into 5 groups ( $n = 8$ ) of mice with tumour volumes of equal mean and range:

1. PBS 0.2ml i.v. Day 0-8
2. HuBC1-g1-M1-MuIL12 20µg i.v. Day 0-8
3. HuBC1-g1-M1-MuIL12 5 µg i.v. Day 0-8
- 5 4. HuBC1-g1-M1-MuIL12 20µg i.v. Every other day, for a total 12 doses
5. HuBC1 Ab 0.5mg i.p. Day 0 and 4 (3 mice only)

Treat evaluation:

Measure tumour size twice a week.

- 10 Determine tumour volume using formula width x length x height x 0.5236.  
Sacrifice any mice having tumour size over 5000mm<sup>3</sup>.  
Calculate T/C ratio (the ratio of treated to control tumour volumes) at the appropriate time points.

### 15 3.2 A431 Subcutaneous Model in SCID CB17 Mice

02-37 Effect of BC1-g1-M1-muIL12 on A431 Subcutaneous Model in SCID  
CB17 Mice

- 20 Mice:  
8 week old SCID CB17 mice, male

Tumour Injection:

- 25 Inject the subcutaneous dorsa in the proximal midline of SCID CB17 mice with  
1 x 10<sup>6</sup> viable A431 tumour cells in 0.1ml PBS following the protocol.

Groups and treatment:

Treatment starts when tumour size reaches ~100mm<sup>3</sup>. Mice are sorted into 2  
groups (n = 8) of mice with tumour volumes of equal mean and range:

- 30 1. PBS 0.2ml i.v. Day 0-7
2. HuBC1-muIL12 20µg i.v. Day 0-7

Evaluation:

Measure tumour size twice a week.

- 5 Determine tumour volume using formula width x length x height x 0.5236.

Sacrifice any mice having tumour size over 5000mm<sup>3</sup>.

Calculate T/C ratio (the ratio of treated to control tumour volumes) at the appropriate time points.

10 3.3 *PC3mm2 Subcutaneous Model in SCID CB17 Mice*

02-44 Effect of BC1-g1-M1-muIL12 on PC3 mm2 Subcutaneous Model in SCID CB17 Mice

15 Mice:

8 week old SCID CB17 mice, male

Tumour Injection:

Inject the subcutaneous dorsa in the proximal midline of SCID CB17 mice with 2

- 20 x 10<sup>6</sup> viable PC3mm2 cells in 0.1 ml PBS following the protocol.

Groups and Treatment:

- 25 Treatment starts when tumour size reaches ~100mm<sup>3</sup>. Mice are sorted into 2 groups (n = 7) of mice with tumour volumes of equal mean and range:

1. PBS 0.2ml i.v. day 0-6

2. BC1-g1-muIL12 20µg i.v. day 0-6

30

Evaluation:

Measure tumour size twice a week.

Determine tumour volume using formula width x length x height x 0.5236.

Sacrifice any mice having tumour size over 5000mm<sup>3</sup>.

Calculate T/C ratio (the ratio of treated to control tumour volumes) at the appropriate time points.

5

#### 3.4 HT-29 Subcutaneous Model in Nude Mice

02-70 Effect of HuBC1-g1-M1-muIL12 on HT-29 Subcutaneous Model in Nude Mice

10

Mice:

6-7 week old nude mice (nu/nu), male

Tumour Injection:

15 Inject the subcutaneous dorsa of nude mice in the proximal midline with  $1 \times 10^6$  viable HT-29 tumour cells in 0.1 ml PBS following the protocol.

Groups and Treatment:

20 Treatment starts when tumour size reaches ~100mm<sup>3</sup>. Mice are sorted into 2 groups (n = 5) of mice with tumour volumes of equal mean and range:

1. PBS 0.2ml i.v. day 0-4

2. HuBC1-g1-muIL12 20µg i.v. day 0-4

25 Evaluation:

Measure tumour size twice a week.

Determine tumour volume using formula width<sup>2</sup> x length x 0.5236.

Sacrifice any mice having tumour size over 5000mm<sup>3</sup>.

30 Calculate T/C ratio (the ratio of treated to control tumour volumes) at the appropriate time points.

*3.5 PC3mm2 Lung Metastasis Model in SCID CB17 Mice*

03-11 Effect of BC1-g1-M1-muIL12 on PC3mm2 Lung Metastasis Model in  
5 SCID CB17 Mice

Mice:

8-week-old SCID CB17 mice, male

10 Tumour Injection:

Inject mice with  $2 \times 10^6$  viable single cells of PC3mm2 in 0.3ml PBS i.v. on  
day 0.

Groups (n = 8) and Treatment:

15

- |                     |                      |
|---------------------|----------------------|
| 1. PBS              | 0.2ml i.v. day 11-15 |
| 2. BC1-g1-M1-muIL12 | 16µg i.v. day 11-15  |
| 3. BC1-g1-M1-muIL12 | 8µg i.v. day 11-15   |

20 Termination:

Sacrifice mice on day 28 or when control mice become sick.

Remove lungs and fix them in Bouin's solution.

Measure lung weight and body weight.

Score lung metastases.

25 Check and record metastases on other organs and lymph nodes.

#### 4. Results

##### 4.1 *U-87MG Subcutaneous Model in Immune Deficient SCID CB17 Mice*

5 First we had to establish a subcutaneous tumour model using the human astrocytoma U-87MG, chosen because of the high level of B-FN expression on this tumour cell line (Mariane *et al.*, 1997, *Cancer* **80**:2378). A titration was done to determine the number of cells to be injected for optimal tumour growth. Different numbers of viable cells (1 to  $6 \times 10^6$ ) were injected into the back of each mouse to  
10 form skin tumours and their rates of growth were monitored (Fig. 5a). Interestingly, regardless of the number of cells injected, the rates of growth of the tumours remained flat for about 3 weeks, after which they all increased rapidly.

For the subsequent experiments,  $4 \times 10^6$  viable cells were injected into the back of  
15 each mouse. Six days later, the average tumour size was about  $135 \text{ mm}^3$ , when treatment was initiated (Day 0). Two groups of mice were treated with 8 consecutive daily i.v. doses of either 5 or 20  $\mu\text{g}$  of huBC1-muIL12. A third group received 20  $\mu\text{g}$  of huBC1-muIL12. i.v. every other day, for a total 12 doses. For comparison, a fourth group of mice received 0.5 mg of huBC1 antibody i.p. on  
20 Day 0 and Day 4. Results of these 4 treatment groups and the control group receiving PBS are shown in Fig.5b. The tumours in the PBS control group grew slowly to  $430 \text{ mm}^3$  by Day 19, by which time the tumours switched to exponential growth, reaching an average size of  $5627 \text{ mm}^3$  by Day 35. Treatment by the antibody had no effect on the tumour growth. Treatment by the different regimens  
25 of huBC1-muIL12 was effective for about 3 weeks in this immune deficient mouse model. By Day 23, the average tumour size of the group treated with 8 daily doses of 20  $\mu\text{g}$  was about  $446 \text{ mm}^3$ , and the average tumour size of the two groups receiving the 80  $\mu\text{g}$  doses was about  $380 \text{ mm}^3$ , as compared to over  $1000 \text{ mm}^3$  for the PBS control group. However, the treatment only delayed the exponential  
30 growth phase by about 4 days, as from Day 23 to Day 35, the tumours in all three groups grew exponentially with a growth rate similar to the PBS treated group.

Table 4 shows average tumour volumes (in mm<sup>3</sup>) of each group on different days.

**Table 4**

Days	PBS	HuBC1-M1- muIL12 (20 µg daily x8)	HuBC1-M1- muIL12 (5 µg daily x8)	HuBC1-M1- muIL12 (20 µg every other day x12)	HuBC1 Ab (0.4 mg daily x2)
0	133.6	134.3	134.8	135.6	144.4
5	163.7	123	135.5	133.4	169.9
8	175.7	91.1	125	113.8	184.2
12	180.3	66.3	115.6	75.9	194.9
15	232	88.8	100	91.9	255.4
19	429.8	215.1	191.9	178.2	425.8
23	1005.5	377.9	446.1	389.3	1059.9
27	2166.6	893.5	1263	976.4	2275.6
30	3474.7	1577.8	2106.2	1658.3	3664.5
35	5626.5	3122.2	3989	3347.4	5847.6

5

#### 4.2 A431 Subcutaneous Model in SCID mice

10 A single-cycle treatment of 7 consecutive daily i.v. doses of 20 µg of huBC1-muIL12 each was effective in the human melanoma A431 subcutaneous model in SCID mice, achieving a T/C ratio of 0.31 by Day 14 and 0.26 by Day 25 (see Fig. 6).

Table 5 shows average tumour volumes (in mm<sup>3</sup>) of each group on different days.

**Table 5**

5

Day	PBS	HuBC1-g1-MuIL12 (20 µg day 0-6)
0	117.8	117.8
3	279.8	205.1
8	632.1	298.7
11	917.3	369.1
14	1390.9	425.0
17	1974.8	506.3
22	3108.1	780.3
25	4238.9	1093.3

#### 4.3 PC3mm2 Subcutaneous Model in SCID mice

- 10 A single-cycle treatment of 7 consecutive daily i.v. doses of 20 µg of huBC1-muIL12 each was effective in the human prostate carcinoma PC3mm2 subcutaneous model in SCID mice, achieving a T/C ratio of 0.34 by Day 15 and 0.33 by Day 25 (see Fig. 7).

15

Table 6 shows average tumour volumes (in mm<sup>3</sup>) of each group on different days.

**Table 6**

<b>Day</b>	<b>PBS</b>	<b>HuBC1-g1-MuIL12</b> (20 µg day 0-6)
0	109.9	109.7
2	224.3	184.7
7	678.8	363.7
9	988.7	435.8
12	1396.0	512.7
15	1777.8	608.3
19	2504.9	805.1
22	3115.8	963.4
27	4058.1	1351.3

5

#### *4.4 HT-29 Subcutaneous Model in Nude mice*

10 A single-cycle treatment of 5 consecutive daily i.v. doses of 20 µg of huBC1-muIL12 each was effective in the human prostate carcinoma PC3mm2 subcutaneous model in SCID mice, achieving a T/C ratio of 0.46 by Day 13 and 0.43 by Day 20 (see Fig. 8). Since it was only a single-cycle treatment and the nude mice lacked functional T cells, it was not too surprising that after Day 20, the rate of growth of the tumours in the treated group started to increase. It will be  
15 interesting to evaluate the benefits of a second cycle of treatment at this time.

Table 7 shows average tumour volumes (in mm<sup>3</sup>) of each group on different days.

**Table 7**

<b>Day</b>	<b>PBS</b>	<b>HuBC1-g1-MuIL12</b> (20 µg day 0-4)
0	81.4	82.5
4	152.1	113.2
7	250.0	143.7
10	317.3	172.9
13	468.1	216.8
17	672.8	286.0
20	837.0	356.9
25	1248.3	570.2
28	1646.8	793.9

5

#### *4.5 PC3mm2 Lung Metastasis Model in SCID mice*

In this xenogeneic model, human prostate carcinoma PC3mm2 cells were injected into severe combined immunodeficient (SCID) mice 11 days before treatment began, allowing ample time for metastases to establish. Despite the lack of functional T and B cells in the SCID mice, 5 daily i.v. injections of huBC1-muIL12 at 16 µg almost completely eradicated the established metastases in all the mice and prevented their outgrowth, as measured by the lung surface covered by metastasis (Fig. 9A) and tumour burden (Fig. 9B). Even the 8 µg dosage was very effective, reducing the lung metastases by about 85%, relative to the PBS control.

Table 8 shows a summary of efficacy data of huBC1-muIL12 in mouse tumour models. The T/C for the subcutaneous (s.c.) tumours is the ratio of average tumour volume of the treated group over that of the PBS control group. For the

20

lung metastasis model, the T/C is the average tumour burden of the treated group over that of the PBS control group.

Table 8

Tumor	Model	Treatment	Dosage	Results	
				T/C	P-Value (vs. ctr)
A431 Epidermoid Carcinoma	s.c.	BC1- $\gamma$ 1-MuIL12	20 $\mu$ g; day 0-6	0.26	0.00038 on day 35
HT-29 Colon Carcinoma	s.c.	BC1- $\gamma$ 1-MuIL12	20 $\mu$ g; day 0-4	0.43	0.076 on day 28
PC3mm2 Prostate adenocarcinoma	s.c.	BC1- $\gamma$ 1-MuIL12	20 $\mu$ g; day 0-6	0.33	0.000039 on day 27
	lung met	BC1- $\gamma$ 1-MuIL12	16 $\mu$ g; day 11-15	0.01 / 0.266	0.0028 on day 27
		BC1- $\gamma$ 1-MuIL12	8 $\mu$ g; day 11-15	0	0.0016
		NHS- $\gamma$ 2h-MuIL12	8 $\mu$ g; day 11-15	0.126 / 0.266	0.0084
U87-MG Astrocytoma	s.c.	BC1- $\gamma$ 1-MuIL12	20 $\mu$ g; day 0-7, 16-23	0.55	0.0027 on day 35
		BC1- $\gamma$ 1-MuIL12	5 $\mu$ g; day 0-7, 16-23	0.71	0.022
		BC1- $\gamma$ 1-MuIL12	20 $\mu$ g; q2d till day 22	0.59	0.012
		HuBC1 Ab	0.4mg; day 0, 4	N/A	0.89

5

## 5. Discussion

The drug candidate huBC1-huIL12 cannot be evaluated in current murine tumour models because human IL-12 is species-specific. Therefore, we produced huBC1-murine IL12 and showed that this surrogate molecule was efficacious in various xenogeneic metastasis and subcutaneous tumour models, as summarised in Table 8. In spite of the fact that SCID mice lacked functional T and B cells, a single cycle of treatment with 7 daily injections inhibited tumour growth by 74 and 67% respectively, in the A431 and PC3 models. Such results are impressive, especially in view of the fact that the huBC1-muIL12 had a very fast clearance rate in the  $\alpha$  phase in mice, relative to huBC1-huIL12 (see Figure 10 and Appendix below). A single cycle of treatment with 5 daily injections was also effective in the HT-29 model in nude mice, inhibiting tumour growth by 57%. Efficacy should improve with multiple-cycles of treatment with huBC1-huIL12 in the clinic, where patients undergoing or post-chemotherapy may have a more functional immune system than the SCID mice. In the PC3mm2 experimental lung metastasis model in SCID mice, 5 daily i.v. injections of huBC1-muIL12 at 16  $\mu$ g nearly completely eradicated metastases which were allowed to establish for

11 days before treatment began.

6. Appendix: Pharmacokinetics of huBC1-muIL12 and huBC1-huIL12

- 5 Pharmacokinetics of huBC1-muIL12 and huBC1-huIL12 were measured in Balb/c mice. It was found that huBC1-huIL12 has a longer serum half-life than huBC1-muIL12 in mice, especially in the  $\alpha$  phase (Fig. 10).

10 **Example 9 – Methods of treatment**

A compound, e.g. fusion protein, of the invention may be used as follows.

- 15 A patient suffering from a cancer, such as glioblastoma, is treated. The preferred route of administration is intravenous or subcutaneous injection, but intramuscular, intraperitoneal, intradermal, or other routes of injection are also possible. Administration by inhalation, orally, or by suppositories is also possible, as are other routes of administration. Administration is preferably in a four-week cycle of three times per week, followed by no treatment for the next three weeks,
- 20 but may be more or less frequent depending on the pharmacokinetic behavior of the BC1-IL12 protein in a given individual. Dosing for an adult of about 70 kilograms is in the range of about 1 to 100 milligrams per dose, with a preferred range of about 4 to 20 milligrams per dose. The most preferred dose is about 10 milligrams for a 70 kg adult treated once per month. Patients are monitored for a
- 25 response according to standard procedures.